THE UNIVERSITY
of EDINBURGH

Muscle factors and demographic characteristics affecting early functional outcome following total knee arthroplasty

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Doctor of Philosophy

The University of Edinburgh

2019

This doctoral thesis is dedicated to Irene and Raie

Declaration

I hereby declare that the work presented in this thesis has been composed by myself alone unless otherwise stated. I have produced this thesis which is submitted for the fulfilment of the degree of Doctor of Philosophy at The University of Edinburgh and has not been submitted for any other degree or professional qualification.

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August 2019

Abstract

Primary total knee arthroplasty (TKA) for end-stage osteoarthritis (OA) is a surgical procedure with a long history of successful outcomes. However, one fifth of patients report dissatisfaction with the outcome of surgery which results from a combination of factors including poor functional improvement. This research examined the functional elements of recovery, with a focus on the role of skeletal muscle. The pain of OA normally limits activity; if the pain is relieved by TKA, any disuse atrophy associated with the OA has been reported in some patients to improve postoperatively with an eventual strengthening. Quadriceps muscle group strength is correlated with activities of daily living (ADLs). Myogenesis is complex but facilitated by muscle precursor stem cells; mainly muscle satellite cells. An individual's skeletal muscle phenotype contributes to their physical recovery following surgery. Many factors have been reported as individually influencing skeletal muscle phenotype, but patient specific clinical data is lacking to evaluate these. In this study, these factors were examined in a primary TKA population to test the hypothesis that specific preoperative and perioperative muscle factors and patient background characteristics affect outcome, with the aim of indicating which patients might benefit from targeted treatment.

Seventy-two patients listed for primary TKA were recruited to a single centre longitudinal observational cohort study. The participants underwent preoperative interview and assessment, provided a quadriceps skeletal muscle sample at time of surgery and completed a range of functional assessments and outcome reports during their recovery until 12 months following their operations.

Muscle biopsies were analysed to determine morphometric and gene expression levels for each patient. Immunofluorescence histology identified muscle fibre type profiles, and quantitative real-time polymerase chain reaction (qPCR) determined relative translational profiles for myogenic, inflammatory, and senescent gene markers. GeNorm methodology identified 3 reference targets as optimal, and EIF4A2, UBC, and GAPDH were noted as the most stable (M=0.55) within the target superficial vastus medialis skeletal muscle tissue in the TKA cohort population. Patient functional performance was evaluated in outpatient hospital research clinics using a battery of functional tests in tandem with patient reported outcome measures

(PROMs) tools. Patient function was also evaluated in a sub-cohort in the community setting using activity monitoring devices.

Different temporal trends were observed between functional measurements. Substantial variation was observed in the response trajectory of functional metrics. Patients perceived that they meaningfully improved earlier than was observed through directly measured functional performance. Compared to preoperative values, by 12 weeks post-op, patients had achieved 82% of final (12 months post-op) PROM score, 63% of final leg power, 63% of final ADL performance, and 5% of final daily step count. The values further increased by 6 months post-op with PROM score increase at 92% of final score, leg power at 94% final, ADL at 116% final, and daily step count at 110% of final value. These findings highlighted the utility of using a combination of diverse metrics to evaluate patient functional outcome and showed that patient evaluated outcome alone may not fully represent early functional outcome. They also emphasise the critical nature of the follow up time points chosen for clinical trials.

Patient background characteristics (demographic, health, and lifestyle factors) were contrasted with patient functional performance and skeletal muscle phenotype using univariate and exploratory multivariate regression modelling. Relationships were identified between patient preoperative baseline factors and post-surgical outcomes, with an observed R^2 range of 0.14-0.31 ($p \leq 0.05$). Associations were found between background characteristics and physiological phenotypes at time of surgery (R^2 0.11-0.29, $p \leq 0.06$). Influences were identified from perioperative physiology on early or primary end-point surgical outcomes, with distinct contributions from certain molecular profiles and skeletal muscle fibre phenotypes (R^2 0.09-0.24, $p<0.05$). Models constructed using patient preoperative function combined with preoperative PROMs parameters provided the strongest prediction of surgical primary functional outcomes (R^2 0.28-0.73, $p \leq 0.006$).

The results provided new insights into the relationship between functional metrics during early recovery post-TKA. The study identified a variety of demographic and muscle related factors which significantly affect patient early surgical outcomes in primary TKA populations.

Lay Summary

Total knee replacement for osteoarthritis (OA) is a surgical procedure with a long history of successful outcomes. However, one fifth of patients report dissatisfaction with the outcome of surgery which results from a combination of factors including poor functional improvement. This research examined the functional elements of recovery, with a focus on the role of skeletal muscle. The pain of OA normally limits activity; if the pain is relieved by joint replacement, the muscle wastage associated with OA has been reported in some patients to improve postoperatively and eventually strengthen. Quadriceps muscle group strength is correlated with activities of daily living (ADLs). The formation of muscle is complex but facilitated by stem cells; mainly muscle satellite cells. An individual's skeletal muscle type contributes to their physical recovery following surgery. Many factors have been reported in laboratory research as influencing skeletal muscle type, but there is a lack of clinical evidence to confirm this in human patients. In this study, these factors were examined in a knee replacement population to evaluate if the factors found before or at the time of surgery affect patient outcome, with the aim of indicating which patients might benefit from targeted treatment.

Seventy-two patients listed for knee replacement were recruited to a cohort study where they would be observed over time at a single hospital site. The participants were interviewed and assessed before surgery, had a muscle sample taken at time of surgery and completed a range of functional assessments and questionnaire reports during their recovery until 12 months following their operations.

Muscle samples were analysed to determine structural and genetic characteristics for each patient. This included the muscle sub-types and sizes, genetic markers of the level of muscle turnover and repair, and genetic markers of the levels of inflammation and cell ageing. Patient functional performance was evaluated in outpatient hospital research clinics using a battery of functional tests in tandem with patient questionnaires. Patient function was also evaluated in a sub-cohort in the community setting using activity monitoring devices.

Different trends were observed between functional measurements over time. Substantial variation was observed in the rate of change of functional metrics. Patients perceived that they meaningfully improved earlier than was seen through directly measured functional performance. These findings highlighted the usefulness of a combination of metrics to assess patient function following knee replacement and show that questionnaires alone may not fully represent patient function. They also emphasise the importance of the choice of follow-up time-points in clinical trials.

Patient background characteristics (demographic, health, and lifestyle factors) were contrasted with patient functional performance and muscle type using statistical modelling. Relationships were found between certain patient characteristics before surgery and how patients recovered. Associations were discovered between background characteristics and muscle sample characteristics at time of surgery. Influences were identified from time of surgery muscle characteristics and how patients recovered. Statistical models made with patient functional performance and questionnaire answers before surgery predicted up to 73% of how they recovered from their operation.

The study results provided new insights into the relationship between patient functional metrics during early recovery following knee replacement surgery. The study identified a variety of demographic and muscle related factors which significantly affect patient early surgical outcomes in knee replacement populations.

Acknowledgements

I would like to thank Prof Hamish Simpson and Dr David Hamilton for their doctoral supervision and introduction to the world of orthopaedic clinical research. Their support and guidance have been instrumental to this thesis and to my doctoral journey. Additionally, the supervisory help and advice of Dr Joanna Brzeszczynska has been fundamental to the molecular scientific aspects of this research study. Her continued supervisory contribution following a move outwith the University of Edinburgh has been laudable and much appreciated.

I would also like to thank the wider orthopaedic research department at the University of Edinburgh for their welcoming atmosphere over the past few years. Specifically, to Steph Collishaw, Dr Robert Wallace, and Dr Helen Handoll for all their advice, and to the wider clinical team at the orthopaedic surgery department in the Royal Infirmary of Edinburgh for all their assistance. I am grateful for the statistical advice from Dr Crispin Jordan and Dr Janusz Brzeszczynski which was incredibly reassuring.

I would like to thank the staff members of the Institute for Academic Development at the University of Edinburgh for their tuition across all academic areas from research methodology to teaching theory. Thanks also goes to the participants of the Muscle Assessed Knee Replacement Outcomes research study at the Royal Infirmary of Edinburgh, without whom there would be no study data.

Personally, this thesis could not have been completed without the extremely valued support of my family and friends. From the simple distractions of conversations, to the breath of fresh air facilitated by very welcome long weekends away, their assistance during my own unrecommended orthopaedic adventure also merits recognition including much hilarity all round from the joys of learning to walk again. All of which I am incredibly grateful for and without which would have made this doctorate a very different experience. Lastly, the greatest thanks of all goes to Emma, who has been my greatest champion.

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Abbreviations

Abbreviation or Acronym	Definition
12M	12 months
12W	12 weeks
18S	18S ribosomal RNA
6M	6 months
6W	6 weeks
Ab	Antibody
ACCORD	Academic and Clinical Central Office for Research and Development
ACL	Anterior Cruciate Ligament
ACS	Acute Compartment Syndrome
ACTB	Actin beta
ADLs	Activities of Daily Living
ADP	Adenosine Diphosphate
Akt	Protein Kinase B
ALF	Aggregated Locomotor Function
AMI	Arthrogenic Muscle Inhibition
ANOVA	Analysis of Variance
ARS	Activity Ratign Scale
ATP	Adenosine Tri-phosphate
ATPase	Adenosine Triphosphatase
ATPSF1B	ATP synthase F1 subunit beta
B2M	Beta-2-microglobulin
BDNF	Brain-Derived Neurotrophic Factor
BEI	Bioelectical impedance
BMI	Body Mass Index
BMP	Bone Morphogenetic Proteins
BOAS	British Othopaedic Association Score
CCI	Charlson Comorbidity Index
CDK2NA	Cyclin-Dependent Kinase Inhibitor 2A
cDNA	Complementary (Synthetic) DNA
CHI	Community Health Index
CIOMS	Council for International Organizations of Medical Sciences
CONSORT	Consolidated Standards of Reporting Trials
CPG	Chronic Pain Grade
Cq	Quantitative Cycle
CRFs	Case Report Forms
CSA	Cross-Sectional-Area
C_t	Cycle Threshold
Cy5	Cyanine 5
CYC1	Cytochrome c1
DAPI	4’,6-Diamidino-2-Phenylindole

DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EIF4A2	Eukaryotic translation initiation factor 4A2
EQ-5D	EuroQol Five Dimension
Eya	Eye absent
FAK	Focal Adhesion Kinase
FAPs	Fibroadipogenic cells
FAS	Functional Activity Scale
FITC	Fluorescein isothiocyanate
FJS	Forgotten Joint Score
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic DNA
GFP	Green Fluorescent Protein
GOI	Gene of Interest
GP	General Practitioner
GPS	Global Positioning System
GUI	Graphic User Interface
HAAS	High Activity Arthroplasty Score
HADS	Hospital Anxiety and Depression Scale
HFKS	High-Flexion Knee Score
HIAR	Heat Induced Antigen Retrieval
HR	Heart Rate
i-NOS	Inducible Nitric Oxide Signalling
ICD	International Classification of Disease
ICOAP	Intermittent and Constant OsteoArthritis Pain
IF	Immunofluorescence
IGF-1	Insulin-like Growth Factor 1
IHC	Immunohistochemistry
IKS	International Knee Society
IL6	Interleukin 6
ILAS	Iowa Level of Assistance Scale
ilSIRENTE	Invecchiamento e Longevità nel Sirente (Aging and Longevity in the Sirente geographic area)
IP67	IP Code, International Protection Marking, IEC standard 60529
IPAQ	International Physical Activity Questionnaire
IRAS	Integrated Research Application System
JAK/STAT	Janus Kinase / Signal Transducer and Activator of Transcription
K&L	Kellgren and Lawrence
KKS	Korean Knee Score
KOOS	Knee Injury and Osteoarthritis Outcome Score
KOS	Knee Outcome Survey
KSS	Knee Society Score
LAM	Laminin
LN_2	Liquid Nitrogen

Loess	Local Regression
MAFbx	Muscle Atrophy F-box, Atrogin-1
MAKRO	Muscle Assessed Knee Replacement Outcome
MEF2	Monocyte Enhancement Factor 2
MEMS	Micro-Electronic-Mechanical Systems
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MODEMS	Musculoskeletal Outcomes Data Evaluation and Management System
MPCs	Myogenic Precursor Cells
MPs	Mesenchymal Progenitor cells
MRF	Myogenic Regulatory (Transcription) Factors
mRNA	Messenger Ribonucleic Acid
MSCs	Mesenchymal Stem Cells
mTOR	Mammalian Target of Rapamycin 1
MuRF1	Muscle RING-Finger Protein 1
MuSC	Muscle Satellite Cells
MWT	Minute Walk Test
Myf4	Myogenic regulatory factor 4
Myf5	Myogenic regulatory factor 5
Myf6	Myogenic regulatory factor 6
MyH1	Myosin Heavy Chain Type 1
MyH2	Myosin Heavy Chain Type 2
MyH7	Myosin Heavy Chain Type 7
MyoD(1)	Myogenic determination factor 1
Myog	Myogenin
NCAM1	Neural Cell Adhesion Molecule 1, CD56
NFkB	Nuclear Factor κ-light-chain Enhancer of Activated B cells
NHP	Nottingham Health Profile
NHS	National Health Service
NIH	National Institute of Health
NJR	National Joint Registry
Nrf2	Nuclear factor erythroid-2-related factor 2
NRS	Numerical Rating Scales
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
NTCs	Non-Template Controls
NZS	New Zealand Score
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
ObsROs	Observer Reported Outcomes
OCT	Optimal Cutting Temperature (compound)
OKS	Oxford Knee Score
OP	Osteoporotic
PAC	Pre-operative Assessment Clinic, Pre-surgical Assessment Clinic

PAR	Physical Activity Restrictions
Pax3	Paired box trasncription factor 3
Pax7	Paired box trasncription factor 7
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline with Tween20
PCR	Polymerase Chain Reaction
PDF	Portable Document Format
pH	Relative activity of hydrogen ions in solution
PICs	PW1+ interstitial progenitor cells
PIS	Patient Information Sheet
POMS	Profile of Mood States
PPE	Personal Protective Equipment
PROMs	Patient Reported Outcome Measures
PSQ	Patient Satisfaction Questionnaire
QC	Quality Control
QOL	Quality of Life
qPCR	Quantitative Polymerase Chain Reaction
R&D	Research and Development
REC	Research Ethics Committee
RIE	Royal Infirmary of Edinburgh
RIN	RNA Integrity Number
ROM	Range of Movement
RPL13A	Ribosomal protein L13a
rRNA	Ribosomal RNA
SAPS	Self-Administered Patient Satisfaction
SDHA	Succinate dehydrogenase complex flavoprotein subunit A
SF-	Short-form-
SIMD	Scottish Index of Multiple Deprivation
SIP	Sickness Impact Profile
SIRT	Sirtuin
Six	Sine Coculus
SOCS3	Suppressor of Cytokine Signaling 3
ssDNA	Single-Stranded DNA
SuRF	Shared University Research Facility
TAS	Tegner Activity Score
THA	Total Hip Arthroplasty
TKA	Total Knee Arthroplasty
TNAP	Tissue Non-specific Akaline Phosphatase
TNF-α	Tumour Necrosis Factor Alpha
TOP1	DNA topoisomerase 1
TRITC	Tetramethylrhodamine isothiocyanate
TUG	Timed Up-and-Go
UBC	Ubiquitin C
UCLA	University of California, Los Angeles

UHPI	Unique Hospital Patient Identifier
UK	United Kingdom
UofE	The University of Edinburgh
UPS	Ubiquitin-Proteasome System
VAS	Visual Analogue Scale
VIF	Variation Inflation Factor
WEL	West and East Lothian
WOMAC	Western Ontario McMaster University Score
WORQ	Work, Osteoarthritis or joint-Replacement Questionnaire
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

Chapter 1: Introduction and Overview

Study Background

One in five patients that undergo knee replacement surgery are dissatisfied with the outcome, amounting to 15,000 dissatisfied patients annually in the UK alone (1),. Dissatisfaction with surgery is largely attributed to unmet preoperative expectations, a lack of pain relief, and a disappointing hospital experience (2). A patient's recovery from surgery and their resulting functional ability are central themes in these factors. Developing further understanding of these areas can eventually allow for a reduction in dissatisfaction.

Short term recovery focuses less on joint health and articulation but instead on the ability of the patient to mobilise, regain independence and gain competence in activities of daily living. The anatomical driver of this function is the level of neuromuscular recovery of the supporting musculature around the operated joint. This research focuses on the muscular aspect of this recovery. Effective recovery of the major weight bearing muscles of the lower limb is key to this recuperation. These muscles have to overcome both wasting from immobility and damage from surgery (3). The focal time of muscular recovery after total knee arthroplasty (TKA) is between six weeks and six months. Effective muscular recovery in this time frame can mean the return to a level of function not achieved for many years. This is frequently seen in osteoarthritic patients who have undergone knee arthroplasty surgery (4).

An influence on this early time within routine clinical practice is that of clinic-based and take-home physical therapy regimes. However even these do not always result in a guaranteed recovery of function (5). The function and power of the quadriceps muscle group are associated with Activities of Daily Living (ADLs) performance during recovery following knee surgery (6,7). The failure of this muscle group to recover function following TKA can result in an inability to perform ADLs and be termed poor surgical outcome. This poor outcome has previously been partially predicted using social, psychological and clinical estimations (8). However, the ability to predict poor outcome may also be improved with a deeper understanding of a patient's muscle

physiology by using quantified physiological markers of muscle regenerative ability. Examples of these markers include growth factors, cytokines, immune responses, and other cell metrics (9). Some of these promising markers relate to the muscle precursor cell, the muscle satellite cell.

Muscle satellite cells are the precursors to myoblasts, myocytes, and myotubes. Much investigation into the cell type in recent years has been performed through *in vitro* study and *in vivo* rodent modelling. Though allowing for high volume of experiments, relatively low research costs and tight control of variables, it is acknowledged that all findings do not directly translate into human physiology (10).

The muscle research that has taken place in humans is largely split between young elite sporting (11) and muscular dystrophy populations (12). These groups have multiple factors that are unconnected to the average primary knee arthroplasty. For example, the sporting population have a clear training history and ongoing training regime, and the dystrophic populations have a clear genetic nature.

With a healthy muscle satellite cell population linked to superior regeneration and performance in mouse models and performance sporting cohorts, substantial investigation of the concept has yet to be performed in a geriatric lower limb arthroplasty population.

Within the population regularly seen for knee arthroplasty surgery, there are lifestyle choices and patient demographics that are closely linked to recovery patterns (13,14). Examples are smoking, high levels of alcohol consumption, a sedentary lifestyle, and obesity. Pre-emptive rehabilitation, or prehab, where strengthening exercises are performed prior to surgery, is widely performed but lacks evidence (15). These factors are yet to be looked at in relation to their effect on muscle satellite cells in a human post-total knee arthroplasty population.

While not routinely an acute life or death scenario, a poor functional outcome following TKA can result in a patient's reduced ADL capability to the point of extreme disability. By identifying patient background characteristics or muscle physiological

targets for therapeutic intervention or clinical care optimisation, the incidence of these outcomes can be reduced in future clinical practise.

Study Overview

This thesis centres around a longitudinal cohort study of osteoarthritic patients who underwent primary total knee replacement surgery. The study aimed to investigate factors affecting surgical outcome, focussing on patient demographics and patient thigh muscle and physiology at time of surgery. Key analytical tools to assess outcome included patient reported outcome measures (PROMs) and direct functional tests. The cohort's muscle samples were also analysed in the laboratory using a battery of investigations.

116 patients were screened for eligibility, 92 patients were approached, and 72 patients agreed to take part in the study. There were 3 cohorts within the study with varying levels of follow-up interaction and data collection. Study data were examined with a focus on patient background characteristics, their relationship to patient muscle physiology, and the physiological relationship of muscle with patient functional outcomes.

Overview of Collected Study Data

Data collection time-points: Surgical Pre-assessment Clinic (PAC: 2-6 weeks pre-op), 6 weeks post-op, 12 weeks post-op, 6 months post-op, 12 months post-op. The categories of collected study data are represented in Table 1.

Table 1 – Overview of collected study data categories.

Data Category	Measurements
Direct Measurements	Leg Power, ALF Score, BMI, BEI, ROM, Daily step count, HR, Sleep quality.
Patient Reported Assessments	OKS, EQ-5D, KOOS, FJS, Pain scores, Complications, Rehabilitation, Experience, Satisfaction.
Muscle Biopsy Laboratory Assessments	Histological (IF): Fibre type, fibre size. Gene expression (qPCR): Myogenesis, inflammation, and senescence groupings.
Patient Baseline Factors	Basic demographics, Vocational history, current and historic activity levels (Tegner), smoking history, alcohol history, orthopaedic treatment history, SIMD quintile, Dominant side, K&L radiographic score, CCI, blood results.

ALF – Aggregated Locomotor Function, BMI – Body Mass Index, BEI – Bioelectrical Impedance, ROM – Range of movement, OKS – Oxford Knee Score, EQ-5D – EuorQol 5 Dimension, KOOS – Knee Injury and Osteoarthritis Outcome Score, FJS – Forgotten Joint Score, IF – Immunofluorescence, qPCR – quantitative polymerase chain reaction, SIMD – Scottish Index of Multiple Deprivation, K&L – Kellgren and Lawrence, CCI – Charlson Comorbidity Index.

Chapter 2: Literature Review

Scientific

Muscle Development and Repair

The musculoskeletal system enables human mobility and autonomy in activities of daily living (ADLs). The failure of skeletal muscle tissue, whether from acute local trauma or systemic chronic pathology, is detrimental to the affected individual.

Early Muscle Development and Repair

Prenatal myogenesis basics

Human skeletal muscle first develops from the dermomyotome within the somite during embryonic development. Precursor cells react to signals from surrounding tissue and commit to the muscle lineage in their anatomical niche (16).

Foetal myogenesis is driven by the Wnt, sonic hedgehog (Shh), and bone morphogenetic proteins (BMP) pathways which regulate the expression of the myogenic regulatory factor transcription factors (MRFs). These initial embryonic muscle precursor pathways and cells differ to the pathways in postnatal muscle tissue (17). Prenatal muscle precursor cells are named myogenic precursor cells (MPCs) and postnatal cells are muscle satellite stem cells (MuSCs).

The MRFs consist of myogenic regulatory factor 5 (Myf5), myogenic determination factor 1 (MyoD), myogenin (MyoG), and herculin also known as myogenic regulatory factor 4 or myogenic regulatory factor 6 (Myf4/Myf6). These factors share functions and govern the process from developmental lineage commitment to proliferation and differentiation. Paired box transcription factor 3 (Pax3), paired box transcription factor 7 (Pax7), sine oculus (Six), and eye absent (Eya) signalling proteins are also involved in the prenatal myogenesis process (18).

This combined pathway transforms MPCs into embryonic and foetal myoblasts that combine to form skeletal muscle fibres, with MuSCs appearing at the end of embryonic development.

Postnatal myogenesis, hypertrophy, and repair is governed by different pathways, with MuSCs and related signalling pathways playing the most important roles in the processes of skeletal muscle tissue construction and repair (19).

Skeletal muscle is the most abundant type of muscle in the body. It is structured into groupings of hierarchical bundles, built up from the basic unit of the myofilaments eventually into groups (Figure 1). Myofilaments perform the voluntary contractions that defines skeletal muscle function in the body. They are grouped into myofibrils, which are grouped into myofibres, which are clustered into fascicles, which make up a muscle. Extensive connective tissue matrices surround each of these groupings, comprised mostly of collagens.

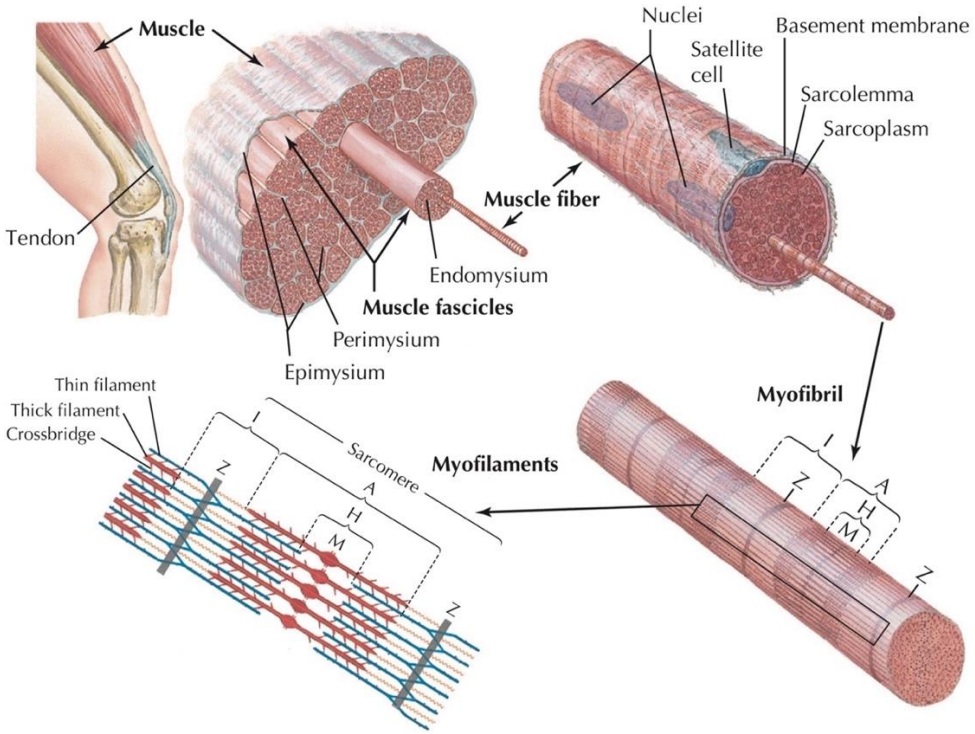

Figure 1 - Hierarchical structure of skeletal muscle. Figure adapted from The Netter Collection of Medical Illustrations(20).

Skeletal muscle tissue is extensively vascularized and innervated to allow for efficient respiration and nervous control.

Nerve action potentials in the somatic peripheral nervous system cause neuromuscular junction depolarization at the motor end plate where the nerve and muscle tissue interface. The depolarization of the neuromuscular junctions is relayed

via the t-tubules and the sarcoplasmic reticulum along the length of each myofibril to allow for simultaneous contraction within each sarcomere unit of myofilaments.

The classical process of contraction, described as the sliding-filament or cross-bridge model is facilitated by several steps, beginning with the depolarization causing calcium ion release which bind to troponin on thin filaments. The thin filaments are anchored to the Z-bands which border each sarcomere. When troponin is bound it reveals neighbouring tropomyosin which allows adenosine tri-phosphate (ATP)-bound myosin heads on thick filaments to bind to actin on thin filaments. Next, ATPase severs ATP into adenosine diphosphate (ADP) and phosphate, with the resulting energy moving the position of the myosin heads. These pull on the actin causing a shortening of the sarcomere unit. The binding of a new ATP molecule to the bound myosin head group releases the actomyosin complexes and allows relaxation.

However, there are some anomalies in the cross-bridge theory of eccentric skeletal muscle contraction and recent findings have identified the prominent role of titin protein in active and passive contraction. The three-filament model helps to explain residual force enhancement, where isometric contraction force is larger following a muscle stretch. This is particularly observed during eccentric contractions. Titin exhibits elastic properties which contribute energy into the sarcomere contraction process in addition to the actomyosin cycles (21–23).

Postnatal human skeletal myogenesis is facilitated in the vast majority of circumstances by Muscle Satellite Cells (MuSCs). Myogenic regulatory transcription factors (MRFs) continue to mediate the process of skeletal muscle repair and hypertrophy in reaction to physical conditioning, damage, or environmental stimulus (19).

MuSCs exist in several states depending on these signals. They can be quiescent and mitotically dormant, they can proliferate, and they can differentiate to fuse into myofibers (24). Figure 2 shows the large heterogeneity of states and expression profiles and lineages of MuSCs.

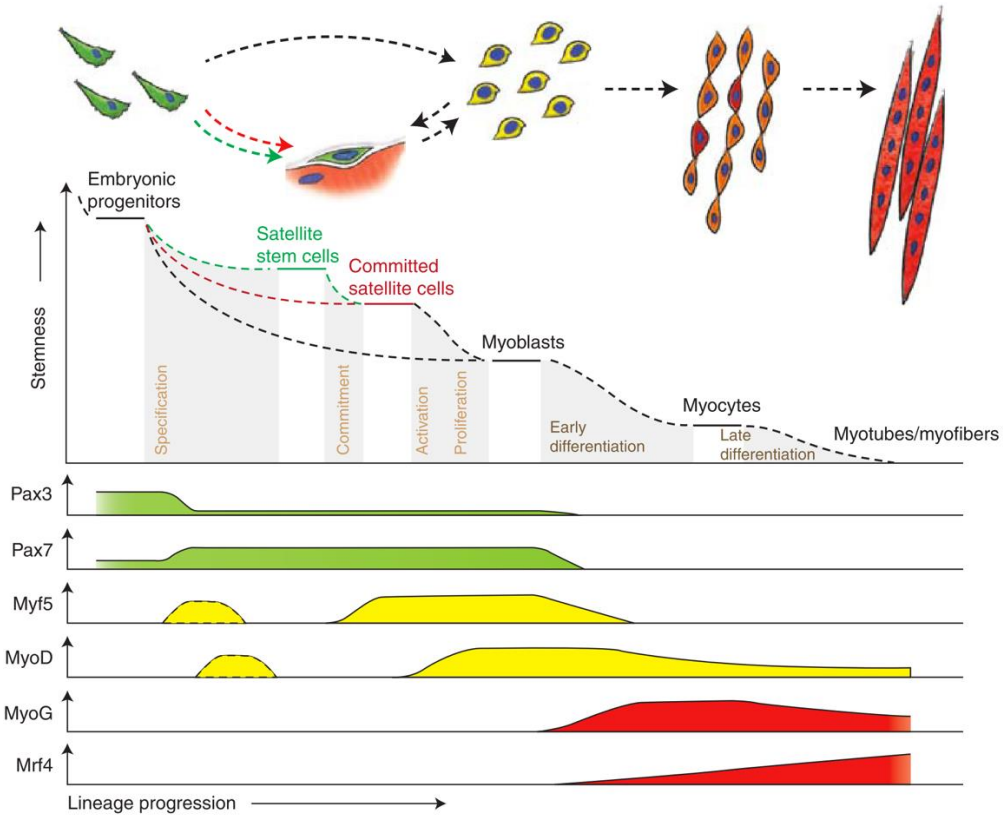

Figure 2 - Muscle satellite cell state variation and lineage progression with corresponding transcription factor expression profiles. Figure adapted from Bentzinger et al 2012 (25).

When proliferating, the cell division axis and polarity determines whether the outcome is two daughter MuSCs, or one daughter MuSC and one cell committed to myogenic differentiation (26–28). Symmetric division serves to replenish the MuSC pool and asymmetric division serves to repair or grow the myofibers. Along with the

MRFs: Myf5, MyoG, MyoD, and Myf4/Myf6, the Pax7 transcription factor plays a major role in the postnatal myogenesis pathway.

Adult MuSCs' can be identified by their transcription factor expression. MuSCs in the quiescent state express Pax7 and Myf5 but not MyoG or MyoD. MuSCs that are proliferating into myoblasts express Myf5 and MyoD. A myogenically committed cell can be identified through the process of fusion and expressing less Pax7, and upregulation of MyoD and MyoG. This myocyte phenotype allows fusion to occur in order to repair the myofibre. Newly formed myofiber segments can be identified by their centrally located nuclei. In healthy muscle, the nuclei migrate to the edge of the fibre and are identical to surrounding undamaged fibres (29).

Thus, markers and molecular analysis techniques can be made to identify MuSCs in their specific states. Pax7 can be used to identify MuSC abundance, MyoD and Myf5 can be used to identify early myogenesis, and MyoG can be used to identify late myogenesis and terminal differentiation into myoblasts.

Myonuclear Domain

Myonuclear domain theory describes how each myonucleus can cover a finite muscle fibre cytoplasmic volume for effective protein transcription. Previously, this volume was thought to be fixed (>25% cross sectional area (30)) beyond which MuSC activity such as nuclear donation was required. However, there is now thought to be more flexibility depending on the nature of the muscle hypertrophy or atrophy (31).

Muscle fibre type is a classification that characterises the composition, phenotype and function of a muscle cell. It is mostly based upon the myosin heavy chain composition that makes up the myosin filaments of the fibre. In humans, the major classifications of fibres are type 1 or type 2, of which there are distinct subtypes (32,33). Type 1 fibres, known as slow-twitch fibres for their relatively slower contraction time, have low glycolytic, high oxidative properties, and are known for their aerobic endurance capacities. Contraction time variances exist due to differences in the time that actin is bound to myosin. Type 2 fibres, known as fast-twitch, contrastingly have high glycolytic and low oxidative characteristics, though

subtypes express variation. Type 2 subtypes include 2A, 2B, and 2X, and overlapping combinations exist resulting from hybrid isoforms of the myosin heavy chain protein (34). Unstained visual differences can be discerned between the two major types due to the difference in myoglobin content, with type 1 fibres having a darker red appearance. There are known differences in force generation between the fibre types; Type 2 fibre types contract with greater force per cross-sectional-area (35).

Fibre hypertrophy can occur without the presence of MuSCs, as demonstrated in some Pax7 knock-out rodent model studies (36). This has reported to be fibre-type dependent and is only witnessed in type-II fibres. Extensive fibrosis was observed in tandem with the fibre hypertrophy. However this has yet to be observed in human studies (36). Current observations indicate that type-I fiber hypertrophy can only occur with differentiated MuSC fusion. This highlights the complexity of the role that MuSCs play within local extracellular matrix (ECM) regulation in coordination with fibroblasts and other cell types.

The muscle fibre type is determined based on developmental processes. Much understanding of the processes has recently been developed through fish models. They are discussed in Rossi's review from 2014 (37), however many of these pathways have yet to be confirmed in humans. Generally, fibre type is based upon developmental regulation involving Sox6, Pbx and Prdm1a transcription factors. Processes also exist to ensure heterogeneity in a muscle grouping for functional purposes. The majority of this occurs during embryonic stages based on genetics and maternal nutrition, but also continues through childhood in response to external stimuli. Fibre typing can also occur to some extent through a fibre plasticity response to specific stimuli during adult life (38). A typical example is the transition from type 1 to type 2X in response to a resistance exercise program, and also from type 2 to type 1 in limb lengthening (39). The signalling pathways for the remodelling process in adulthood are outlined in Bassel-Duby's review from 2006 (40). Innervation is required for plasticity changes, and muscle fibres default to type 1 in response to denervation. A muscle group typically has more than one fibre type but different muscle groups are comprised of different fibre type ratios due to their varied

functions (41). A group involved in holding body posture will have a greater percentage of oxidative fibres.

Differences in the molecular composition of each fibre type can help to identify each isoform. Type 1 fibres express myosin heavy chain type 7 (MyH7) protein, type 2A express myosin heavy chain type 2 (MyH2), and type 2X express myosin heavy chain type 1 (MyH1) (34).

Historic studies have shown that elderly individuals show reduced muscle mass and changes in their fibre compositions (42). This typically takes the form of a reduction in the size and percentage of type 2 fibres. This has been used as a marker of aged muscle compared to young, however it does not correlate with all humans once a certain age is reached. For example, not all individuals over 50 years old display the same phenotype, but the average at age 70 shows reduced mass, particularly amongst type 2 fibres, compared to the average 20-year-old (43). Other factors are therefore thought to contribute, such as genetics and lifestyle choices, leading to differences in chronological and biological age at this stage in life (44).

Myogenic Progenitors – A more complex picture

Other cell types (as well as MuSCs) have recently been identified as playing direct and indirect roles in the adult skeletal myogenesis. These include pericytes, PW1+ interstitial progenitor cells (PICs), fibroadipogenic (FAPs) also known as mesenchymal progenitors (MPs), CD133+ cells, muscle side population cells (MPs), and myoendothelial cells. Some of these have been suggested as having potential in cell therapeutics as the properties of MuSCs change *ex-vivo* which limits their use (45). Additional endogenous cells such as myeloid cells, endothelial cells, and fibroblasts also can contribute towards related signalling pathways (46).

Pericytes have been observed playing a role in the myogenesis process. A pericyte subpopulation expressing tissue non-specific alkaline phosphatase (TNAP) has been observed contributing to skeletal muscle in mice (47). These pericyte-derived mesoangioblasts have been observed contributing to the satellite cell pool during regeneration *in vitro* (48). However, when investigated with human clinical trial cell

transplantation, they had low efficacy with the cell lineage failing to manifest (49). The contributions of these pericytes in humans is yet to be directly observed.

FAPs or MPs are different to mesenchymal stem cells (MSCs). They can contribute under certain circumstances to myogenesis. They have been termed “promyogenic” as their function in controlling the extra-cellular matrix is also key to the repair process (50). *In vitro* FAPs have been observed to change into myoblasts, but this has not been observed in humans (50).

PICs stand out amongst the other promyogenic cell populations as also contributing directly to the satellite cell pool. They originate from a different lineage from embryonic MuSCs and can differentiate into MuSCs and adipocytes (48).

The resulting interactions between each of these cell populations and the exact role each plays in myogenesis still requires much elucidation. However, as the diverse nature of signal expression amongst and between these groups is further determined, the accepted cross-over between distinct cell types and differently described subpopulations of MuSCs themselves may change as further understanding is gained (48).

Despite variations in lineage, the vast majority of myogenesis is accepted as facilitated by MuSCs.

Muscle Damage, Disease and Ageing

Muscle damage can be due to trauma or acute pathology, resulting in abrupt losses of muscle mass and function. Slower muscle atrophy can occur from other pathology or simply from disuse. Overall, muscle atrophy occurs due to greater muscle protein breakdown than protein synthesis. This imbalance can result from a variety of scenarios.

It is thought that the down regulation of transcription signalling pathways are the main cause of disuse atrophy (51). Key pathways are the insulin-like growth factor 1 – protein kinase B – mammalian target of rapamycin 1 (IGF-1-Akt-mTOR) pathway and the focal adhesion kinase – protein kinase B – mammalian target of rapamycin 1 (FAK-Akt-mTOR) pathway. The mechanical chronic unloading of muscle leads to the reduced activation of both synthesis pathways leading to a reduction in protein generation (51).

Increased muscle degradation is a component of this negative balance of cell and protein turnover. The ubiquitin protease system is the most proteolytic, with calpains, caspase-3, and the autophagy-lysomal system also contributing. The ubiquitin E3 ligase muscle RING-finger protein-1 (MuRF1) and muscle atrophy F-box (MAFbx/atrogin-1) are both strongly expressed during muscle atrophy and are frequently used as molecular markers to detect this (51).

Trauma and Muscle Tissue Breakdown

Traumatic musculoskeletal damage has a range of causes. These range from chronic overexertion to acute mechanical force damage (52), which in the extreme can lead to complications culminating in tissue necrosis and amputations (53). In these circumstances, grafting muscle tissue from one body are to another can help to salvage viability (54).

Additional factors that cause muscle damage include chemical toxin exposure, extremes of temperature, infection, and autoimmune disorders (55). Even electrolyte

imbalances such as phosphates, potassium, and sodium in the blood can lead to cases of rhabdomyolysis or striated muscle breakdown (56).

Autoimmune idiopathic inflammatory myopathies, with an annual incidence of 1 in 1,000,000 have been reported to occur when environmental exposure is combined with a defective immune response (57,58). This leads to self-recognising immunogenicity through a variety of mechanisms (59). Resulting myositis causes muscle weakness and, in some subtypes, necrosis of muscle fibres.

Orthopaedic procedures cause damage in the process of intervention. For example, arthroplasty operations by nature cause trauma to the target joint. Surgical approaches have been developed to minimise collateral tissue damage but the nature of accessing a joint makes this unavoidable. For total knee arthroplasty, a variety of approaches exist for differing reasons (60). The medial parapatellar approach is popular in the UK. It involves cutting into the joint capsule along the patellar ligament and quadriceps tendon to access the joint (61). This approach has been compared to the theoretically less invasive subvastus knee arthroplasty surgical approach as it avoids the knee extensor mechanism, however both have the same functional outcomes following the initial few post-operative days. This has been shown in both a randomised controlled trial with patients undergoing single leg total knee arthroplasty operations, and in patients undergoing bilateral procedures with one knee randomised to each approach (62,63). Understanding the muscle physiological changes caused by TKA trauma are relevant to contextualising the post-operative recovery process.

Muscle injury and repair has multiple stages. Initially, the broken myofibers and ensuing inflammatory response form a hematoma. Any dead tissue is digested by phagocytic cells and myofibre repair is facilitated by MuSC-derived myoblast integration. New capillarisation may take place depending on the extent of injury, and any connective tissue scarring is modified while muscle function is re-established (64).

The Ageing Process: Elderly Skeletal Muscle

Comorbidities such as cancer, hypertension, obesity, and organ failure are more prevalent in the older population and are found in the elective arthroplasty population.

Cachexia and Sarcopenia

Cachexia is defined as weight loss, inflammation, and emaciation due to chronic disease. In patients, it may also present as weakness and fatigue. Frequently, this is from cancer, organ failure or rheumatoid arthritis (65). At least 50% of cancer patients show symptoms of cachexia, of which 80% are dead within one year (66,67). Historically, cachexia has been the greatest cause of death in cancer patients (68,69). Tumour growth leads to protein degradation in skeletal muscle as alenine and glutamine are used by the tumour. The increased quantities of lactate production from glucose by the tumour are also massively energy detracting and inefficient (70).

Sarcopenia

Sarcopenia develops in the elderly due to a range of factors (67). These include anabolic signalling blocks, muscle breakdown through the calpain, UPS, and autophagy pathways, mitochondrial functional turnover blocks, and redox homeostasis. Sarcopenia is thought to be the biological mechanism behind elderly physical frailty (71). Past the age of 50 years old, annual skeletal muscle wasting occurs at roughly 1.5% per year. Once a level of reduced mass and function is reached, as defined by clinical scores, a diagnosis of sarcopenia can be made (72).

A factor thought to contribute to sarcopenia is reduced MuSC number in old age as demonstrated in Kadi's study from 2004 (73). The study's sample size was robust (young n=31, elderly n=27) but the single muscle group choice (tibialis anterior) may have limited these findings. Some transgenic mouse models have shown hypertrophy in MuSC null elderly mice, but the question of translatability from these mouse studies to the human remains (74,75). Hypertrophy typically occurs here through expansion of the myonuclear domain, which may not be sustainable. Elderly mice are defined as roughly 2 years old compared to human studies where the average patient

is several decades older. Additionally, criticisms have been made that mice confined to a cage do not represent normal physiological conditions (76), and of methodological problems with the models' selection of the tiabialis anterior muscle group to study (77).

Circulating myostatin, which inhibits myogenesis, is a controversial cause of sarcopenia. Opposing studies measuring levels following bouts of resistance exercise have disagreed: though Snijders' study used more robust modern laboratory techniques (78), Ratkevicius's study was more robust with a sample size three times larger (79). The larger study showed no difference in levels between young and old, however Snijders' study showed delayed expression of MuSC content. Other biomarkers have been proposed such as, IL-6, SOCS3, and potentially TNF-α, but due to the phenotypic variation there is no consistent panel for diagnosis of sarcopenia (71,76).

More recent findings seem to indicate that rather than the raw number of the MuSCs, or even the number of MuSCs per fibre or fibre type, that it is the MuSC state regarding propensity to activate or self-renew that may differ in old age (80). Change in the local milieu can dictate polarity and therefore the division criteria (81).

The effect of diet on patient MuSC function is poorly understood, but some evidence points to supplementary amino acid ingestion increasing function (82,83). Certain aspects of diet have been observed (primarily in animal models) to affect both the immune system as well as the musculoskeletal system (84). Particularly seen in studies focussing on training regimes but may have future potential to aid recovery in the orthopaedic clinic.

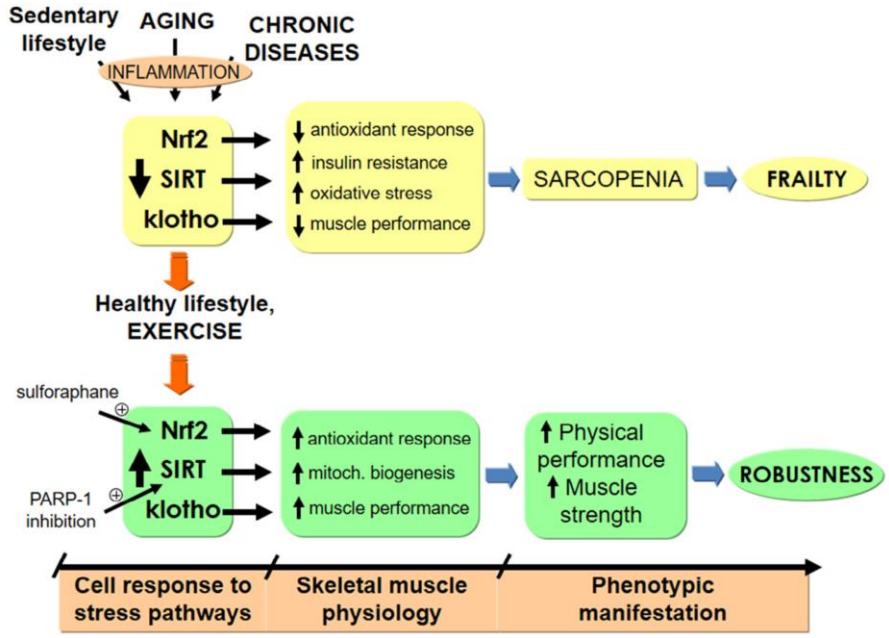

Figure 3 – Diagram of cell signalling pathways leading to sarcopenia with demonstration of the interventional effect of exercise. Figure from Angulo et al (85). *Nrf2 - nuclear factor erythroid-2-related factor 2, SIRT – Sirtuin, mitoch – mitochondrial.*

The physiological response to exercise is frequently used in research to compare these variables. The influence of exercise on the nuclear factor erythroid-2-related factor 2 (Nrf2), sirtuin (SIRT), and klotho is thought to counteract the natural decline of the expression of these factors due to ageing and sedentary lifestyles (85). Increasing exercise reduces oxidative stress and increases mitochondrial biogenesis through the upregulation of these factors (Figure 3).

The elderly have smaller skeletal fibre cross-sectional-area (CSA) compared to younger individuals, particularly in type 2 fibres (86–88), as well as lessened response to hypertrophic stimuli. They also have less fibres, once again particularly type 2 fibres (89,90). These aspects of atrophy and hypoplasia are well established. High-intensity resistance exercise in osteoarthritic elderly men has been found to be more effective than in women, but still more effective than moderate-intensity resistance exercise (91). The ability of individuals with OA to undertake hypertrophic exercise programs is limited by kinesiophobia (avoiding movement due to fear of pain) (92). Following TKA, this has been observed to continue to be a relevant factor in return to activity (93,94). The exact mechanisms continue to be investigated with cognitive and behavioural factors as likely candidates (95). In this context, the exact fibre-specific changes following TKA are yet to be explained.

Oxidative capacity is related to the vascularisation of the tissue. Therefore, the extent of capillary networks of skeletal muscle can indicate the endurance capacity of an individual. A correlation between reduced capillarisation and normal ageing has proved contentious. Some studies show lower capillarisation in the elderly in conjunction with hypertension and sarcopenia (96,97), but others show similar capillarisation in the elderly compared to young controls (98). Barnouin's study, examined very active elderly individuals which may well have skewed the findings and does not represent a typical arthroplasty population.

Overall, telomere shortening, where chromosomes are mitotically truncated resulting in replicative senescence, does not occur in skeletal muscle ageing, and is likely not a factor in sarcopenia as reviewed by Lorenzi et al (99). However, the current studies have methodological limitations concerning the nature of a clinical frailty group, the invasiveness of biopsies and the heterogenous cell content of typical patient samples. As a result of these shortcomings, it has been recommended that single whole fibre investigations are performed to confirm the findings of a lack of telomere shortening. Thus, the effect on MuSCs has been hard to quantify in elderly and frail individuals (100). Murine models with induced shortened telomeres have been shown to have no detrimental effect on MuSC function (101).

Pathology: Osteoarthritis and Muscle

End stage osteoarthritis (OA) is the primary indication for total knee arthroplasty (TKA). Primarily affecting older individuals, osteoarthritis has different pathogenic causes and stages, as highlighted in Figure 4. Initial risk factors for developing OA include demographic and biomechanical factors. These include sex, race, obesity, and joint injuries, and can be metabolic or post-traumatic. The incidence of some risk factors naturally increases with age resulting in a higher prevalence of OA in the elderly. Typical disease progression begins with damage to joint cartilage past the point of possible repair leaving subchondral bone exposed (102). Osteophytes grow at the edge of joint surfaces, and changes can lead to thickening of the joint capsule. This leaves a narrowed joint space which typically causes discomfort and pain to the

individual. OA is more prominent in weight bearing joints such as the knee and hip compared to upper limb joints.

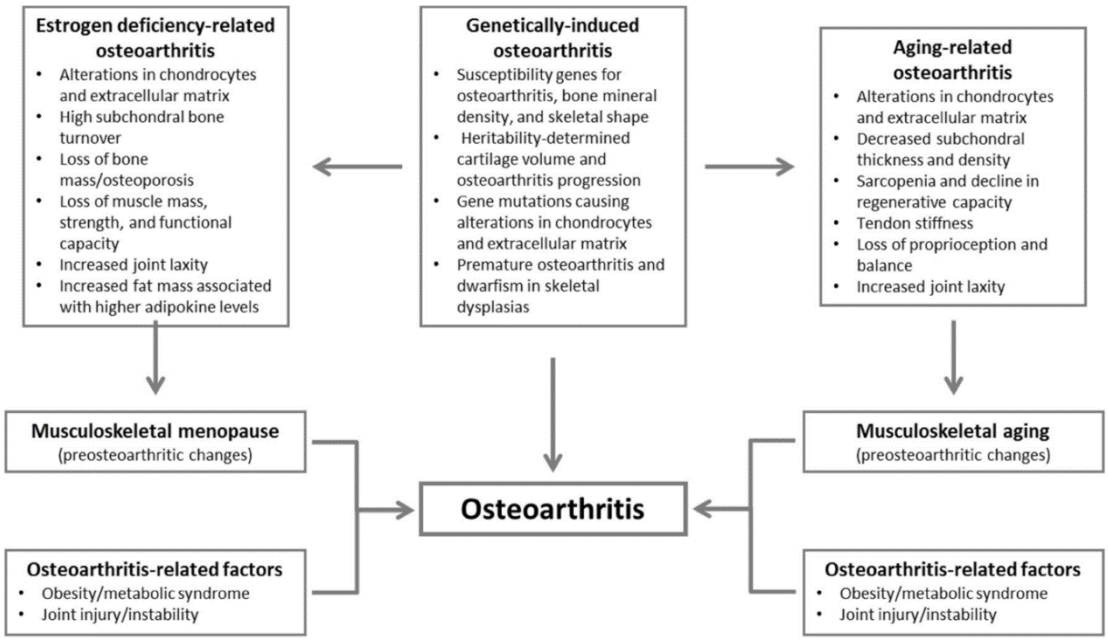

Figure 4 – Summary flow-chart showing the pathogenic causes and phenotypes of the three subtypes of osteoarthritis. Figure from Bruyère 2015 (103).

Cartilage repair is facilitated by chondrocytes. Trauma, hormonal changes, and genetic predispositions can change their phenotype and accelerate the detrimental effects leading to OA (103). Biomechanical factors such as increased weight can also contribute to this, with early clinical treatment commonly including weight management and physical activity advice.

OA is a complex condition with no current cure (104). Much research is currently ongoing to target the pathogenic causes and to elucidate mechanisms further (105). TKA is the primary treatment for end-stage OA once early conservative interventions have been exhausted.

It has previously been suggested that selective atrophy of hamstrings lead to the quadriceps becoming dominant and that this can lead to OA. However, the techniques used to investigate this lacked depth (106), and did not take the confounding factors of age and BMI criteria into account in the analysis (107). Greater muscle atrophy has been linked to OA severity and, similarly, atrophy of quadriceps has been observed in those with patellofemoral joint OA (108). However, these study

results seem to indicate disuse atrophy could be mostly accounted for by reduced joint movement because of pain.

With a projected ageing population, and a mean age for primary TKA of 68, the UK is poised to see a rise in OA and similar age-related musculoskeletal diseases over the coming decades (Figure 5) (109). Anticipating this potential rise in incidence, research into these diseases and conditions is pertinent to improving healthcare.

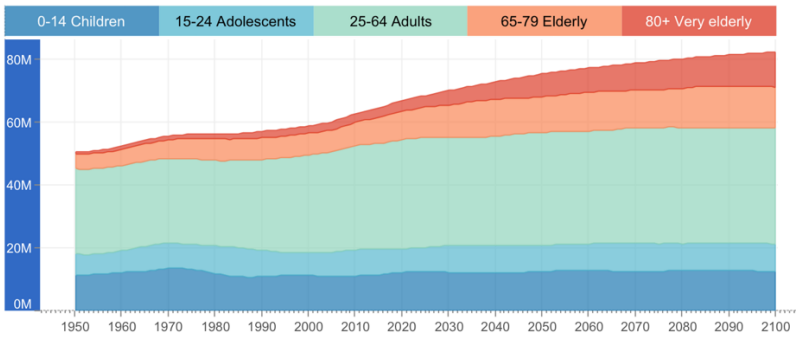

Figure 5 - UK population historic breakdown and projection over the next 80 years. Adapted from UN DESA 2015 (110,111).

As an interventional procedure in response to a disease, it is important to determine if arthritic processes have any paracrine effects on skeletal muscle functions, or vice-versa. The cross-talk interactions are discussed in depth in Krishnasamy's review from 2018 (112). She identified potential relationships include myokines blocking inflammatory pathways of chondrocytes, where disruption of secretion leads to inflammation and increased pain from neurotrophins such as brain derived neurotrophic factor (BDNF) (113). The release by muscle cells of pro-chondrogenic IGF-1 may regulate cartilage development and upkeep. On the other hand, OA and associated symptoms may impact on skeletal muscle function. For example, the adipose infiltration of skeletal muscle reduces strength and fibre recruitment in OA patients (114,115). An overview of the levels of signalling is presented in Figure 6.

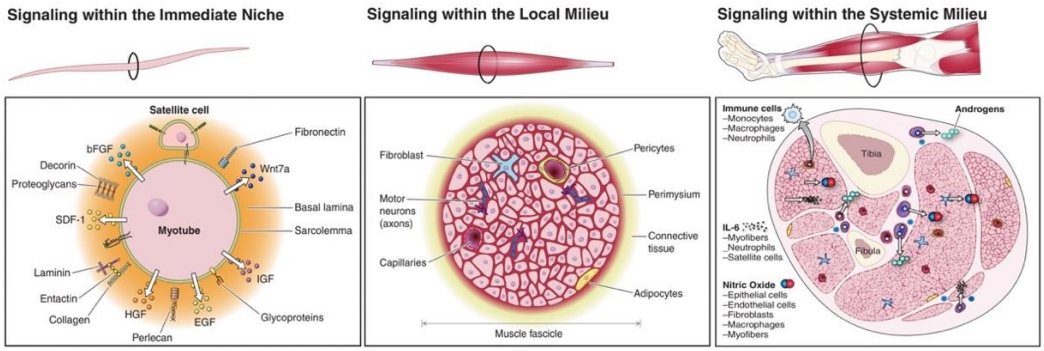

Figure 6 - Overview of the levels of intercellular and inter-tissue signalling related to skeletal muscle. Figure adapted from Yin et al 2013 (24).

These signalling effect levels span (a) the immediate niche, (b) the local milieu and (c) the systemic milieu. Cell-to-cell signalling of growth factors can direct MuSCs into mitosis in the immediate viscinity of muscle fibres. Within a muscle fascicle, the interactions between more diverse cell types occur at a slower rate but with different purpose. The systemic milieu includes endocrine hormones and the wider immune system, all of which can impact upon MuSC activity (24). This range highlights the diversity of the MuSC local and wider environments, and the complexity regulating their activity.

A recent investigation into disease specific effects on skeletal muscle function targeted clusterin protein expression in osteoporotic (OP) and osteoarthritic human populations (116). Clusterin, a protein related to inflammation and senescence, was found to have high expression in OP patients but not in OA patients. However, other published studies have previously shown mixed results regarding clusterin correlation with OA (117–119). In these, clusterin deposits have been found to correlate with muscle fibre degradation, and clusterin protein immunoreactivity has been found to associate with muscle denervation atrophy (120).

The use of some pharmaceuticals associated with pain management of OA has been known to impact upon MuSC function. Non-steroidal anti-inflammatory drugs (NSAIDs) impair the function of MuSC through blocking of prostaglandins and growth factors which prevent the fusion of myoblasts (121). However, it seems the effect may be more pronounced in young cohorts as observed in young athletes (122). Differences have additionally been observed between oral and intramuscular infusions, with the effect only being seen in oral doses (123). A small but bilaterally controlled cohort was used in the infusion study, which may have impacted results due to sample size. Nevertheless, this may suggest more complex factors are involved. The exact influence of NSAIDs on MuSC function has yet to be clinically confirmed.

Concern has been raised about the inability to activate musculature surrounding the operated joint after an arthroplasty procedure (3,124). Rather than a pain related issue, this effect is mostly attributed to joint effusions affecting sensory nerve

receptors and is termed arthrogenic muscle inhibition (AMI) (125,126). AMI is associated in OA knee patients with increased pain and decreased bone marrow legions (127). The loss in muscle strength loss has been postulated to be more influenced by denervation than by muscle autophagy (128).

The rehabilitation post-joint-replacement can take different forms ranging from an immediate post-op assessment along with a self-guided exercise sheet, to regular post-hospital-discharge outpatient appointments. A study examining the differences between patients who made use of the two extremes in TKA and THA populations found that those attending regular sessions post-discharge performed worse than those who did not (129). Some of those making use of the latter service had self-referred and others had been referred by their General Practitioner, though the proportion is not specified. Despite the therapeutic benefit of physiotherapy intervention, results indicate that a skewing may have occurred towards those already with poor outcomes. It was not possible to pre-operatively predict physiotherapy need. The results indicate that patients struggling with function are referred by themselves or by a clinician to physiotherapy, however most patients without issue do not make use of the service. While a patient's MuSC content cannot directly influence PROM response or hospital satisfaction, the compounding effects on muscle phenotypes and resulting function within an outcomes window may have a modifiable effect on these headline outcome parameters.

Diabetes mellitus is a comorbidity present in at least 10% of the knee arthroplasty population (130). While acknowledged as being at larger risk of infection post-surgery due to circulatory factors (131), little consideration is given to the condition's effect on MuSCs and related muscular recovery following arthroplasty. Recent type 2 diabetes murine model studies have been conflicted, some showing no-effect, as well as some showing delayed or reduced MuSC function (132). This reduced function presented in content or proliferation rate. More research is required with an emphasis on translational studies to understand this issue further.

Across all pathology, negative impact on MuSC and skeletal muscle function may be impacted by inflammatory and senescence pathways (133,134). Identification of the

presence of these markers can be compared to the presence of functional impairment and potential causative factors. Once senescence MuSC pathways are activated and systemic, they can be irreversible with fatal consequences. This can occur through the de-repression of as CDK2NA (also known as $p16^{INK4a}$) locus resulting in pathway upregulation as investigated in a model study by Sousa-Victor (135). Markers of senescence can therefore be identified by analysing expression of CDK2NA and of tumor necrosis factor alpha (TNF-α), an inflammatory cytokine. Markers of inflammation can be investigated through identification of interleukin 6 (IL-6), an inflammatory cytokine. However, analysis is complicated due to its co-role as a myokine with controversial evidence pitting a reported positive impact on MuSCs against its role as a promotor of atrophy (136). Despite this controversy, it is well used as a marker of negative inflammatory function in molecular analysis of muscle samples (137).

The nuclear factor κ-light-chain enhancer of activated B cells (NFκB) transcription factor has also been identified as playing a role influencing myogenesis at various stages(138). TNF-α and IL6 can induce NFκB signalling which can lead to muscle wasting, especially in the presence of inducible nitric oxide signalling (i-NOS) (139). The Monocyte enhancement factor 2 (MEF2) grouped factors have been implicated in muscle remodelling in ageing (140). They therefore can be said to cooperatively increase the activity of myogenic transcription factors in models.

Research modelling skeletal muscle factors *in vitro* and *in vivo* does not always translate to human clinical relevance (10,141). Bareja's 2014 flow cytometric species comparison study showed the differences in mechanisms well (142). Specifically, they found differences in genetic myogenesis, in cytokine sensitivity, and in cell interactions. Mouse MuSCs differentiate more readily than human MuSCs, and to a different lineage of daughter cells. The mouse MuSCs also reacted positively to some inflammatory cytokines whereas human MuSCs responded negatively.

Human musculoskeletal research is typically focussed in three areas: namely congenital and developmental areas, young active populations (11), and muscular dystrophy patients (12). The clinical niches are quite distinct. However, skeletal muscle is an important component to consider in orthopaedic patients. Orthopaedics research has focussed upon surgical technique, bone biology, and biomechanics to optimise the implant components for function and longevity. The muscle recovery component of arthroplasty is under-researched.

Sporting populations have a clear training history and ongoing programs, and the dystrophic populations have a genetic nature with a physiological focus different to the normal population. Additionally, particularly with Duchenne's muscular dystrophy, patients show a satellite cell population favouring stemness. This is the reverse of that seen in an aged population, typically seen in arthroplasty cohorts, of increased differentiation and a scarcity of stem cells (27).

The training history of elite sporting individuals, particularly that including resistance exercise, biases them towards contemporaneous fibre type profiles and towards hypertrophy in later life. Skeletal muscle myonuclei acquired from training persist long after the stimuli have been removed (143). This long term effect is also seen in doping cases with performance enhancement agents, such as anabolic steroid use, where benefits are seen for decades following initial use (144). A recent model has attempted to disprove this finding, but the methodology of the study contained very few mice (minimum 6 per experimental group), chose a muscle group known for lacking translatability (145), and took place over only a few months (146), making the

results unconvincing. The exact effect of life-long exercise on elderly MuSC content is yet to be elucidated but the mid-term effects following a short resistance exercise program suggested that MuSC changes are unsustained (30).

As humans age, the skeletal muscle physiological response to exercise becomes dampened (147). In parallel, the dietary effect of reduced protein absorption and muscle-protein synthesis can compound the weakened effects of strengthening exercises (148,149). However, the state of MuSCs has been hypothesised to influence the response rate to these exercise stimuli and may be used to identify those that respond better to exercise rehabilitation programs (150). Comparison of these phenotype data with recording of individuals' historic exercise and activity habits may identify behavioural patterns that are linked to favourable functional recovery post-TKA.

With a healthy muscle satellite cell population linked to superior regeneration and performance both in mouse models and performance sporting cohorts, there remains to be substantial investigation of the related physiology in an elderly lower limb arthroplasty clinical population.

The use of a pneumatic tourniquet to control blood loss during surgery has been postulated to influence quadriceps function. Routinely used in the UK during TKA, though global use varies, it has been associated with elevated risk of distal deep vein thrombosis (151). The effect from applying this pressure during surgery in theory can affect the MuSC niche and function during the very early recovery phase. A study with a primary end point of 48 hours post-surgery found no difference in leg strength (152), however this time-point is may be too early to determine the full effect on functional outcome. Another study investigated the effect until 12-months post-op found quadriceps inhibition and greater pain during the first 6-months of recovery in their tourniquet study group (153). However, the exact effect on the MuSC niche is yet to be elucidated. Perhaps an analogous scenario is that of blood-flow restriction exercise training, where an artificial anaerobic environment is created to enhance the training stimulus. This has been shown in a small cohort to enhance short term MuSC function following training in this manner (154), however the ischemia

reperfusion injury from a pneumatic tourniquet may be to a greater magnitude. Blood-flow restriction exercise enhances the MuSC proliferation through the increase in phosphorylation of $p70S6K^{Thr389}$ and elevated $p38MAPK^{Thr180/Tyr182}$. The transcriptional profile of vastus lateralis muscle following TKA using tourniquet was compared to that from a non-tourniquet group and found to show upregulation of cell stress pathways (155).

Patient Background and Osteoarthritis

Patient background characteristics have previously been associated with OA onset and with surgical outcomes post-TKA to varying extents. These characteristics range from associations with demographic factors to lifestyle choices.

Retrospective studies have shown associations between certain occupations and OA onset (156–158), however concern has been raised about publication bias, with a resulting recommendation for inclusion as a research metric in future prospective cohorts (159). Occupations involving high-load manual labour and kneeling are closely associated with knee OA, however evidence suggests genetic inheritance involvement when evaluating frequently hereditary manual occupations such as farming and fishing (160).

Overall, the evidence for some of these attributes conflicts, with more research required to determine full relationships and underlying causes.

Clinical

Introduction to Orthopaedic Surgery and Recovery

Joint replacement, also known as arthroplasty, as a treatment for osteoarthritis is an elective operation with incredibly successful outcomes. Diseased or damaged bone joint surfaces are removed and replaced with articulating synthetic components. In 2017, 90,000 total knee arthroplasties were performed in England and Wales (109). Arthroplasty of the hip and knee joints in particular can lead to pain relief and large improvements in quality of life for patients (161). However, knee replacement components are susceptible to wear and tear deterioration. Current clinical techniques and prosthetic designs for total knee arthroplasty have been reported to have a 95% survival at 20 years with 82% of replacement operations lasting 25 years or more (162). Inevitably, components or their fixtures break down requiring revision surgery to replace the components (163). This is typically more complicated and variable than a primary arthroplasty due to the variety of causes, wear patterns, and failure methods (164,165).

Total knee arthroplasty is a surgical procedure requiring a major incision, arthrotomy, bone resection, and substantial endoprosthesis fixation. The surgery itself seeks to ease pain and increase function in a patient. While the observed end surgical outcome at 1-2 years post-operation is typically positive, short term recovery can be variable. One in five patients that undergo the surgery are dissatisfied with the outcome at 12-months post-op (1), amounting to over 18,000 dissatisfied patients annually in the UK alone. Dissatisfaction with surgery is largely attributed to unmet preoperative expectations, a lack of pain relief, and a disappointing hospital experience (2). The preoperative expectations aspect of dissatisfaction includes patients' own expectations of functional outcome following surgery alongside other elements such as subsequent pain levels.

Short term recovery focuses on the ability of the patient to mobilise, regain independence and achieve competence in activities of daily living (ADLs). Which is largely dependent on the musculature around the operated joint.

Effective functional improvement of the major weight bearing muscles of the lower limb is key to this recovery. These muscles – mostly of the hamstrings and quadriceps groups (3) – may have to overcome both wasting from recent immobility and damage from surgery. The main period of muscle recovery after total knee arthroplasty surgery is between six weeks and six months (166). Effective muscular recovery in this time frame can mean the return to a level of function not achieved for many years (4).

Recovery from Total Knee Arthroplasty

Immediate post-op ward care is aimed at facilitating a safe and speedy discharge. These approaches are different from the historical post-arthroplasty clinical pathway of prolonged bedrest (167,168).

The current early rehabilitation within routine clinical practice is that of clinic-based and take-home physical therapy regimes. However these do not always result in a good recovery of function (5). The function and power of the quadriceps muscle group are associated with ADLs performance during recovery following knee surgery (6,7). The failure of this muscle group to recover function following TKA can result in an inability to perform ADLs and be termed poor surgical outcome. This poor outcome has previously been partially predicted using social, psychological and clinical estimations (8). However, the ability to predict poor outcome may also be improved with a deeper understanding of a patient's muscle physiology, given the phenotypic variation in this area, by using quantified physiological markers of muscle regenerative ability. Examples of these markers include growth factors, cytokines, immune responses, and cell metrics (9). Some of these promising markers are related to the functional status of the muscle precursor cell, the muscle satellite cell (MuSC).

In 2013, Hamilton et al found that the number of satellite cells found in a patient's quadriceps muscle group were more predictive of outcome than preoperative lower limb power measurements (169). Intra-operative muscle satellite cell count was identified as a better predictor of post-operative leg power improvement than pre-operative leg power. This pilot followed two separate cohorts containing 17 and 11

patients who underwent total knee replacement for osteoarthritis. Methods used for analysis were an evaluation of satellite cell content with immunohistochemistry and qPCR, and power assessment with a Nottingham Leg Extensor Power Rig. The laboratory analyses limitations included a small sample size, lack of replication, and at times unclear results.

The cells identified by Hamilton et al (2013) as the best predictors of functional power improvement were the muscle satellite cells which are the precursors to myoblasts, myocytes, and myotubes. They can be defined as muscle stem cells, though show some variation in phenotype. Discovered in the early 1960s (170,171) and named based on their anatomical location at the periphery of muscle fibres sandwiched between the basal lamina and sarcolemma. They form a heterogeneous population with differing propensity to remain quiescent, mitotically proliferate, or differentiate into terminally differentiated daughter muscle cells. Recent summary reviews of the satellite cell niche evaluate many aspects of current understanding of the physiology of the cell type and the role they play (29,172–174).

Investigating their function in human knee arthroplasty populations can could be of benefit in optimising patients' recovery post joint replacement.

Clinical Research Outcomes Measurements

Clinical outcomes are measures of patient health status used within clinical practice, clinical research, and in service quality processes (175,176). They range from a simple note of a patient symptom or laboratory result to other measurements and can be based upon psychometric and statistical analyses. Measurements can include technical recordings, patient interviews, or direct observations.

These can be clinician or researcher led evaluations, known as Observer Reported Outcomes, usually recorded in physical or electronic Case Report Forms (CRFs) in clinical research or Medical Notes in routine clinical practice. The measurements can alternatively be recorded by patients themselves, termed Patient Reported Outcomes (PROs) (177).

Most human evaluated methodologies, excluding the operation of certain technical apparatus, have elements of subjectivity. However, technical equipment data retains elements of operator variation. Variation can be reduced through appropriate training, standardisation by standard operating protocols, and consistency in the same individual recording data where possible (178).

To allow comparative assessment between cases or of recovery progression, data must be succinctly documented. Evaluations of physical assessments, and charting progression of physical ability contemporaneously in the clinic environment can provide clear information into this (179–181). However, overly frequent onsite assessment of patients within Research Hospital settings can quickly become a burden to both patients and facilities (182,183).

When patient data collection is planned more frequently than local routine clinical appointment schedules, other data collection methods must be utilised. Research-specific clinical departments or remote data acquisition is necessary. Frequent repeat visits to a hospital site can create patient burden, leaving remote acquisition as more suitable. Modern techniques allow for various methods for collecting this (184). These methods include implantable and wearable devices, alongside other traditional patient contact methods. Device recorded data can be uploaded at

intervals during clinic visits, or with constant wireless data transmission back to a computer server at the research hub. Patient contact methods, traditionally via postal letter or phone call, now include options such as batch emails, text messages, web portal data entry, and video calls. All these data collection methods have security considerations and strict confidentiality policies which must be understood and adhered to (185).

The standardised recording of patient data in clinical cohort research creates ease of analysis and comparisons. Analytical methods exist for comparative analysis of qualitative metrics, though they can present complex limitations in large cohorts. Semi-quantitative or quantitative data are preferred as they can be easily collated and interrogated with direct statistical analyses (186). Choosing data categories for collection can be challenging during study design. Clinical trials or cohort studies can take place over many years and the chosen metrics must be set at the study outset. To aid selection, many established direct and surrogate measurements exist across various categories within the musculoskeletal research literature (187).

When assessing post-operative function, similar metrics can provide different levels of sensitivity and insight. While the validation process for different outcome measurements determines these factors, the use of multiple types of assessments can help to complete the clinical picture. Where possible, this can be achieved through the use of both functional outcomes and PROMs when assessing patients(188).

Patient Reported Outcome Measurements (PROMs)

Patient reported outcome measures (PROMs) are subjective reports of a patient's health or experience provided by the patient themselves and without interpretation by a health professional (189). Routinely collected within the UK's National Health Service (NHS) since 2009, they are now a core component of both routine clinical practice quality assurance and in clinical research (190).

PROMs encompass subjective experiential feedback from patients. Relevant topics within musculoskeletal research include clinical care experience, functional experience such as activities of daily living (ADL) competence, and activity specific pain levels.

Methods for recording PROM data are varied. They encompass many responses to enquiries regarding their symptoms and clinical concerns. PROMs can be recorded face-to-face with patients in a clinic scenario as part of a consultation interview or remotely in several ways. In both scenario types, a standardised format must be followed to ensure reliability. Some methods require more researcher input than others, and comparisons of recording methods show variation in patient preference and response rates (191). For example, phone calls are individual and require patient convenience, mailed questionnaires are efficient with time but have low response rates, and web database input forms have low response rates but remove the latter need for analogue to digital response conversion pre-analysis (191). The most consistent recording method is in-clinic where possible. Participant focus is ensured, with the ability to provide clarity or context if necessary.

A transition from paper-based to electronic PROMs format has recently become preferred due to increased efficiency and reliability in recording (192). Care is taken in the transferral to electronic versions to ensure equivalence is demonstrated between formats (193,194). While the format of electronic PROMs tools have previously been confined to proprietary tablet devices linked to a centre or study, the recent emergence of application-based reporting mechanisms that work from any internet-connected device has opened new avenues for academic research (195).

Assessment of the Knee Osteoarthritis Patient Population with PROMs

The use of PROM tools in the assessment of total knee arthroplasty populations is well established (196). These vary in length, dimension scope, target audience, language, and history. Some are proprietary, some are free to use.

A list of prominent PROMs related to total knee arthroplasty are listed in Appendix A: Clinical Study Documentation from adapted information in published reviews (197,198).

Recent research using PROMs tools in TKA populations has investigated factors ranging from health status, to satisfaction, to work status, to the changes in ability to perform activities of daily living following surgery (199–201). The tools are utilised to examine aspects of routine TKA outcome to determine how current clinical procedures or service provision affects patients, and could be adapted (190). They are also used to determine differences between existing surgical treatments and novel endoprosthetic designs in clinical trials (63,202).

Variation in comorbidities, patient biometrics such as body mass index and in patient demographics have been shown to affect Oxford Knee Score (OKS), a PROM tool specific to evaluating knee symptoms and function, following TKA (203,204). The comorbidities data originated from over 2700 patients, making them very robust for a single study site.

Case mix describes the range of patient background and health status of those being admitted for a surgical procedure. This can include patient age, sex, comorbidities, and other factors. The range of patients admitted to a hospital can identify “cherry picking” of favourable cases and therefore affect the overall rate of positive outcomes for a department or hospital. Previous research in the United States identified that TKA outcomes are consistent for low-risk patients across all types of hospital settings, but high-risk patients have better outcomes at smaller speciality centres (205). However, they also identified elements of “cherry picking” in the case selection at those centres. International comparisons of TKA case mix outcomes have found national differences in outcomes, pointing to potential differences in

physiology and in cultural context during recovery from TKA (206,207). The suggested cultural contextual factors included differences in routine intervention time-point in late stage OA and ranging positions during post-operative recovery.

Examination of outcome using PROM tools has identified the importance of baseline scores of some metrics in relation to post-operative outcomes. The EuroQol Five Dimension PROM tool (EQ-5D), measuring health-related quality of life, has been shown to increase post-operatively by similar amounts for each clinical BMI category (204). Patients showed worse pre-operative scores with increasing baseline BMI. Patient PROM scores improved post-operatively, but each category showed similar increases resulting in similar variance in post-operative outcomes.

Just as surgical case mix can confer different outcomes for surgeons' individual annual lists, the case mix of study recruitment can influence outcomes in research. While inclusion and exclusion study criteria help to narrow variation and control for confounding factors, recruitment has inherent variation due to the factor of case mix.

Patient hospital experience has been shown to affect outcomes following TKA. Outcomes measured using the OKS and the Short Form-12 (SF-12) PROM tools showed that those with poor hospital experience had outcome scores less than half of those who described their experience as excellent (208). However, the assessment of hospital experience was assessed at 6-months following operation and may have been influenced by patient early outcome when retrospectively evaluating hospital experience. This is addressed in the study paper's discussion as recall bias.

Observer Reported Outcomes

Observer reported outcomes are based upon a variety of factors. Examples of these include raw biometric measurements, discussions with patients, data from specialised instrumentation, and physical assessments. The latter two categories can be targeted to specific clinical groups.

They are distinct from PROMs as they must be recorded by a clinical or research professional to record them. Elements of subjectivity can still exist within these outcomes.

Functional Assessment of the Knee Osteoarthritis Patient Population

To measure functional outcomes following TKA, there are bodies of validated, well-used tests in the literature (Table 2). Functional assessments exist that are validated for specific clinical populations (209). These are known as performance-based measures and can target both OA patients and those recovering from major surgical knee operations.

For a functional test to be considered robust, it must be assessed for accuracy and reproducibility. This includes inter- and intra-operator reliability, test interpretability, and test responsiveness, amongst other criteria (210). The composition of validation tests varies.

Published comparisons and best practice recommendations for OA functional outcome assessment tools are intermittently published by OARSI, the Osteoarthritis Research Society International, from the consensus of a large number of experts in the field. Some of these focus on tests specific to knee OA function. A seminal publication recommended a timed up-and-go test, a walk test, a stair climb test, a chair to stand test, and a duration walk test as a best practice combined testing battery to assess post-TKA function (211). These tests do not require specialised expensive equipment or require large amounts of space to be conducted. Another publication assessed intra- and inter-operator variability of these tests with a large patient population and found them to be robust (212).

Table 2 - List of example functional performance-measures for assessing lower limb osteoarthritic patients. Adapted from OARSI (211).

Identified activity themes	Identified tests
Walking short distances	Fast-paced walk test – 50 ft
	Fast-paced walk test 40 m (4 × 10)
	Fast-paced walk test 40 m (2 × 20)
	Fast-paced walk test 80 m (8 × 10)
	Self-paced 13 m
	ALF battery
	Self-paced walk test 8 ft
	Multi-paced 5 m
Walking long distances	Six MWT
Getting in/out of a chair	Timed up and go test
	Get up and go test
	ALF battery
	Chair-stand test – 30 s
	Chair-stand test – five repetition
Rising from a stool	1-leg rising
Getting in and out of bed	Steultjens battery
	ILAS battery
Rolling over in bed	ILAS battery
Turning whilst walking (ambulatory transitions)	TUG
Walking down/down stairs	Stair-climb test –12-step
	Stair-climb test – nine-step
	Stair-climb test – four-step
Hopping	1-leg hop test
Kneeling	
Standing up from sitting on floor	
Full squat on two legs	Maximum squat test
Semi squat on one leg	1-leg squat test
Static standing balance	FAS battery
Reaching in standing	Functional reach test
Putting on socks/footwear	Sock test
Getting in/out of a car	PAR battery
Running	Fig 8 running test
	Zig–zag run test
Lifting and carrying objects	PAR battery
	Steultjens battery
Walking on different surfaces	
Walking around/over obstacles	
Stair negotiation	Nine step stair test
	Twelve step stair test
	Four step stair test
	ALF battery

FAS, Functional Activity Scale; PAR, Physical Activity Restrictions; ILAS, Iowa Level of Assistance Scale; ALF, Aggregated Locomotor Function Score.

Recognition has also been made of the differences between PROMs and directly measured functional data (213). While only evaluated at 1-month and 12-months post-op in Mizner et al.'s study, his observations indicate they may follow differing trajectories. Further research from his group indicated that in the first month following surgery, patients perform better in PROM scores than in functional scores but subsequently follow a collinear trend (188,214). The exact improvement rate during early outcome time-points following TKA is yet to be fully understood. Concerns have previously been raised about using PROMs measurements alone, however the use of stronger psychometric evaluation techniques and the flexibility of electronic PROMs technology have improved data quality (215). When well-

constructed, PROMs tools can be as robust as objective, directly measured functional measurements to determine patient outcome (216).

Biometric measurements include the range of movement of a joint, body mass index (BMI), and bioelectrical impedance (BEI). Measurements of the limb circumference such as a thigh can be used to assess muscle mass, however evidence for validity of this metric is mixed (217,218). While a non-invasive quick measurement, aspects of body fat and identifying a reproducible anatomical location on the patient's thigh create uncertainty. Unreliability has been reported in obese patients (219), and the measurement has also been reported as susceptible to sex differences (217). The technique is not as accurate as magnetic resonance imaging (MRI) to determine muscle mass (220), but can provide a quick and cost effective in-clinic measurement that can comparatively assess atrophy and indicate potential underlying sarcopenia (221).

Dual energy X-ray absorptiometry is considered reliable for the assessment of muscle mass but exposes a patient to a very low ionising radiation dose, which may be deemed an excessive burden within research by an ethical review board. Bioelectrical impedance analysis can be used non-invasively to assess muscle mass and is frequently used to assess sarcopenic populations (222–225). Body mass index assesses weight to height ratio. A study of early outcomes found BMI had no influence on functional recovery following TKA, however it was conducted in a relatively small sample size and patients were evaluated at a maximum of 12 weeks following surgery (226). The impact of pain could still be a major factor in some patients at this stage of recovery. Another study showed those with obese BMI ratings had significantly reduced functional recovery compared to those with lower BMI scores (227). Separately, higher BMIs have been shown to influence some PROMs scores but not others, with patient surgical satisfaction most susceptible to negative influence from high BMI (204).

Wearable Electronic Health Monitoring Devices

New technological approaches to remote data capture provide interesting and novel avenues for data collection.

Wearable technology is a trend developing with increasing speed over the past decade. It is a fast-paced competitive commercial area which provides incredible opportunity to healthcare research (228). Areas of clinical relevance which occur away from the clinical environment can now be remotely and constantly monitored. Insight which previously required specialised and cumbersome equipment can now be obtained from discreet wearable devices. Concerns about privacy are rightly placed, but, with appropriate oversights and regulation, the use of such devices provides valuable insight going forward in both clinical practice and clinical research (185,229–231). These regulations include considerations to ensure appropriate confidentiality when using and transferring patient data, and related accountability to the hospital or NHS trust's patient data authority.

Take-home health monitoring devices have a history spanning decades with several notable categories of relevance. These include cardiovascular, activity, body temperature, galvanic skin response (conductivity), and blood oxygen saturation monitoring devices (228). Some aim to cover multiple categories of these in a single device. The creation of micro-electronic-mechanical systems (MEMS) has allowed for the miniaturisation of health monitoring instrumentation, with what began in aerospace industry now transitioned to consumer devices in the community (232,233).

Activity Monitoring Devices for TKA Clinical Research

There are now a large variety of styles and functions to choose from when selecting a wearable electronic health monitoring device.

The use of health monitoring devices for monitoring TKA outcomes dictates desired device attributes. The average TKA patient is 68 years old and has pain and mobility issues. While comorbidities are frequently present, examining mobility as a primary assessment characteristic is relevant when assessing function. Within the health sensor sector, these instruments are known as activity monitoring devices. They collect data primarily on user movement and can range from simple accelerometer-

based step-counters, to GPS-connected devices with activity-specific insights that incorporate a large number of sensors into a single compact system.

Metrics are based upon distinct sensors, which rely on algorithmic analysis to categorise raw data into defined class intervals or bins (234). Examples of sensor categorisation include step cadence, heart rate, workout 'zones', estimations of calorific expenditure, and sleep times, quality and depth (231). Activities categorisation ranges from time spent sitting, to the number of steps taken, to the smoothness of a swimming stroke (235).

Activity monitoring device systems can attach to the body of the subject in different ways. Some are wrist-based, others are placed in a garment pocket, attached to the waistband, or fixed directly to the skin and wrapped in a dressing to secure them. Each style balances accuracy with convenience. The popular clinical research device ActivPal requires gluing to a subject's thigh (236). The devices are validated for a variety of clinical study uses, however the collected data is limited, and the devices are inconvenient compared to other application methods (236). The developers have recently introduced a pocket-based device, however this application method is susceptible to missing data due to garment removal (237). Wrist-based devices provide similar accelerometer data to those previously used on the waist or thigh, and can also provide further patient information through additional sensors such as heart-rate monitoring (238,239).

Previous research by Luna et al utilised wrist-based activity monitoring devices as part of an outcomes tools battery to assess TKA patients in the three weeks following surgery (240). The study identified that during the first month of recovery, TKA patients reported perceived improved function prior to a measured increase in activity. However, methodologically, they only retrieved 80% of their target activity monitoring data, and only recorded activity for 14 hours per day, which may have skewed their findings. The devices also measured generic proprietary activity (in actigraphy units unique to the device which lacked definition), and a direct surrogate to step count could not be made, as the readings could have been confounded by upper limb movement (241).

The measurement of patient daily step count in the community provides useful insight, but the relationship with health status is yet to be defined. It has been stated that a routine daily count over 1000 steps is beneficial compared to a completely sedentary lifestyle of under this amount, however the subsequent higher categories and their health implications remain contested (242). Factors such as age and comorbidities vastly change observations as a healthy target for a 20 year old is expected to be greater than an 80 year old individual post-TKA (243). Suggestions for healthy step count targets range from 3000-10000 steps. However, after lower limb arthroplasty or in patients with a comorbidity, the target is roughly 30% lower (242). The levels and duration of moderate-to-vigorous physical activity are also important for cardiovascular health, however this metric is more relevant to younger individuals (244). In addition, walking cadence is a relevant metric which provides further context to daily step count. An observational study focussing on these metrics by Webber et al found similar step counts and cadences between patient post-TKA and those awaiting surgery (244). However, counts for the TKA patients were lower compared to accepted numbers for healthy individuals. The comparisons were performed in separate cohorts and only at one time-point for each cohort, averaging 34 participants each. The use of literature healthy control data instead of collected study data devalued this secondary comparison, however the recordings were made over 7 days and the device was reliable which made the measurements in their study robust.

Many activity monitoring devices have graphic displays which present a live tracking of their activity. The ability to observe these numbers has been assessed a motivational tool during early recovery post-TKA and post-THA. In a study of 163 patients with 5% loss to follow-up, greater step count was observed in the first 6 weeks post-op and at 6-months post-op in those who were not blinded (245). KOOS and EQ-5D scores were similar between the cohorts at 6-months post-op. The study design and combined TKA and THA cohort provided potentially confounding factors, as the proportion of TKA:THA patients was not identical in the blinding and unblinded cohorts. In addition, there were other demographic discrepancies between the cohorts. The conflation of set step count targets and blinding to progress also

combined two potentially influential factors. The motivational factors relating to unblinded activity monitor provision without stated step targets remains unclear.

Additional activity categories can be recorded by monitoring devices such as heart rate, sleep quality, and metabolic energy expenditure. Wrist-based heart rate measurements, taken once every minute with optical photoplethysmography, are accepted as reliable at rest (246). However, concern remains over the effect of stimulants such as caffeine as an uncontrolled variable when measuring resting heart rate in a study population (247). Measurement of sleep qualities result from algorithmic binning of accelerometer data during defined evening, nighttime, and morning time periods. They provide insight into duration of sleep, and proportions of light or deep sleep. Lighter sleep is defined as restless, encompassing relatively more movement (e.g. turning in bed). Patient pain and comorbidities are known to influence sleep quality and duration (248), which are known to vary in a post-TKA cohort (249). The data category of metabolic energy expenditure, usually recorded in calories is based upon large assumptions made on fitting primary step count and heart rate data into formulae based on average general population biometrics (250). As a result, it is not a reliable wrist-based activity monitor metric for use in clinical research.

Preoperative Prediction of TKA Outcomes

The identification of associations between pre-operative baseline data and patient outcomes following TKA provides an opportunity to identify at-risk groups of patients prior to their operations (251). Factors predicting outcome, provide important targets for pre-operative optimisation and can facilitate targeted recovery-care planning.

Previous research has attempted to predict patient surgical outcomes with a combination of preoperative measurements. These predictions are estimated with PROM tools, functional scores, patient background factors, or other combinations of pre-operative metrics. Regression statistical modelling or correlation coefficients are typically used to evaluate predictive relationships (252). Some research groups have attempted to develop individual pre-op tools that try to predict early outcome post-

TKA, for example the Prediction Rule by Lungu et al which identifies at risk patients (8).

Several studies have investigated predictive factors of 6-month outcome following TKA. One study identified that surgical outcomes at 6-months post-op could be pre-operatively predicted by the Oxford Knee Score PROM tool, pre-op chronic pain level, pain expectations, and coping abilities (253). Similarly, factors such as poor pre-operative function and social isolation have been shown to predict poor 6-month outcome (254). In another study, a prominent finding was the relationship between pre-operative and post-operative function (255). The results inferred that the optimisation of the identified factors prior to surgery would improve outcomes. It was suggested that the post-op outcomes could be improved by preoperative activity modification or by reducing the progression of OA and its associated decline in function. Some believe that prehabilitation can overcome negative effects by improving a patient's functional strength and performance before an operation, but recent findings indicate the limited potential of prehabilitation regimes in TKA patients (15,256). Neuromuscular electrical stimulation has also been examined as a recovery tool for TKA patients (257). Study results showed short-term quadriceps activation improvement but no long-term effects.

Functional elements have been extensively discussed in the literature as direct predictors of functional outcome following TKA, however there is not a consistent relationship, with different factors identified depending on primary outcome or time-point (255,258–260). For example, those with higher surrogate ADL functional performance pre-op do not always perform the best in similar tests post-op, with psychosocial elements previously identified as negative influences on outcomes post-TKA (261–263).

Pain levels pre-op have been shown to be predictive of post-op pain scores, however these have mostly been performed during very early time-point in the first few days after surgery (264).

Patient background factors such as side dominance, historical activity preference, and use of tobacco and alcohol have been examined to determine associations with outcome post-TKA.

The side preference of leg dominance is associated with superior fine motor control and biomechanical stability in certain tasks compared to a non-dominant side (265). The factor has been investigated in the OA arthroplasty literature but not conclusively due to small cohorts (266,267). In these studies, the superior motor control, stability, and confidence in a dominant leg has been reported to translate into marginally improved functional outcomes compared to TKA in a non-dominant leg.

The correlation between an individual's sporting history and OA is varied, with recent evidence pointing to high intensity and high frequency sports being a potential causative factor of OA (268,269). The inferred mechanism includes a higher propensity to traumatic joint injury and a likelihood to return to activity prior to full recovery, thereby compounding damage. However, the quality of evidence across many studies in this area has been evaluated as poor (270).

Amongst the population regularly seen for knee arthroplasty surgery, there are lifestyle choices that are closely linked to negative surgical recovery patterns (13,14). Examples are smoking, high levels of alcohol consumption, a sedentary lifestyle, and obesity. These factors are yet to be looked at in relation to their effect on satellite cells in a human arthroplasty population.

A recent retrospective study in over 100000 patients examined the effect of smoking on surgical outcome post-TKA and found that it was associated with a higher rate of complications, use of analgesia, and mortality, but PROMs remained similar to non-smoking groups (271). Consumption of alcohol and use of tobacco has been shown to reduce in OA patients following TKA (272), however those continuing to drink alcohol or smoke tobacco showing higher levels of periprosthetic joint infection (273). No link was found between hospital length of stay or readmission rates for those with an alcohol use history, however greater readmission rates were found within 1 month post-op for tobacco smokers (274).

These studies highlight how certain lifestyle choices increase the risk factors for OA progression and also affect the outcome following TKA. Examining these factors in relation to muscle physiological profile may provide important context to underlying mechanisms of poor outcome.

As studied previously, preoperative symptom severity, is a relevant predictive factor. However, arthritis severity and its relationship with knee extensor power or strength is conflicted. Many studies show the factors are inversely related (as reviewed by Zwart et al in 2018 (275)), with higher OA severity and progression linked to weaker knee extensor torque, particularly in women. However, when adjusted for knee compartment alignment, this effect disappears (276).

Few studies have evaluated the associations and predictive effect of physiological markers on surgical outcomes. Perioperative synovial fluid examination identified higher TNF-α concentration levels were correlated with subsequent lower pain and better function at 6-weeks post-op. It was postulated that the TNF-α levels were associated with inflammation in the joint capsule. Arthroplasty was considered to remove this stimulus resulting in a reduction in pain (277). Conversely, another study investigated the outcomes 2-years post-TKA. They found that lower TNF-α levels in synovial fluid were associated with better outcomes (278). The latter study only evaluated outcome with the WOMAC index and did not use direct functional tests. The evaluation of stress hormones in patients' urine prior to surgery was investigated as a predictor of 3-month post-operative pain. Higher catecholamine and cortisone levels pre-op were found to be predictors of lower post-op pain, in opposition to the study's initial hypothesis (279). The observations are described as complex due to changes in pre-operative activity such as a reduction due to fear of pain, and to varying levels of anticipatory stress.

A recent study investigating post-TKA muscle atrophy and the effect of essential amino acid supplementation found that diet in the week prior to surgery was associated with the levels of inflammatory markers as a prediction of atrophy in the immediate two weeks following the operation (280). Previous pilot work by the same

group had identified pre-op diet to be associated with stronger performance in 6-week post-op functional tests (281). However, a longer outcomes measurement window is required to confirm if the effects persist. In addition, the laboratory techniques require additional quality steps to improve data robustness.

Associations with gender and preoperative activity score have been used to predict post-operative activity levels by Turnbull et al (282). Additional observations included an observation that higher social deprivation predicted lower post-op activity, health status, and patient reported outcome. They additionally found that younger patients were more likely to be dissatisfied with surgery.

Expanding the scope of pre- and peri-operative factors to include physiological factors related to the musculature surrounding the target joint of the TKA procedure may help to improve the models for predicting outcome.

Aims and Research Hypotheses

The study's Primary Hypothesis was:

- Preoperative and perioperative muscle factors in conjunction with patient background characteristics predict early functional outcome following primary total knee arthroplasty.

Patient characteristics and peri-operative muscle physiology were compared to their early functional surgical outcomes following primary total knee arthroplasty. The study's Primary Aim was therefore:

- To identify preoperative and perioperative muscle factors and patient background characteristics affecting early functional outcome following primary total knee arthroplasty.

The study methodological structure required laboratory and clinical investigations of an observational longitudinal patient cohort. As such, the primary aim required several steps to examine the concept within a total knee arthroplasty population. Initially, background characteristics and baseline factors were defined. Then, the functional outcomes were measured at various time-points. Finally, relationships between background, baseline and outcome factors were examined. The patient characteristics and factors were defined from distinct categories of demographic, biometric, comorbidity, and lifestyle choices.

An observational longitudinal cohort study was established with five time-points to evaluate temporal trends in functional outcome. Once established, the factors affecting early functional outcome were examined using sequential comparisons to understand the relationships. Initially, individual factors and outcomes were analysed to establish correlations. Following this, multivariate analysis was used to provide insight into the complex associations between background characteristics, muscle physiology, and surgical outcomes:

1. Patient characteristics was first compared to early surgical outcomes.
2. Patient skeletal muscle physiology to surgical outcomes.
3. Patient background characteristics to patient skeletal muscle physiology.

4. Finally, multivariate modelling of patient skeletal muscle and physiology factors was compared to surgical outcomes to identify combined relationships.

The study's Secondary Hypotheses are therefore:

- Patient background characteristics correlate with functional surgical outcomes following primary total knee arthroplasty.
- Patient muscle physiology at time of surgery correlates with functional surgical outcomes following primary total knee arthroplasty.
- Patient background correlates with patient muscle physiology at time of surgery in patients undergoing primary total knee arthroplasty.

While ultimate final recovery is the lasting impact of the surgical intervention, large variance is observed within patient populations during the very early recovery stage, up until 6-months following the surgical intervention. The rate of recovery does not always correlate with final functional outcome.

The study's Tertiary Hypotheses were therefore:

Tertiary hypotheses:

- Patient background characteristics and muscle factors affect very early functional recovery following primary total knee arthroplasty.
- The pattern of recovery of different categories of outcome metrics vary during the early recovery stage in primary total knee arthroplasty population.

The same statistical stepwise methodology was used to examine both very early functional outcome and primary outcome at the study's final time-point. Temporal trends were examined and the relationships between the categories of outcome measures were assessed. The time-point at which the different outcome measurements plateau was also defined.

Chapter 3: Longitudinal Clinical Cohort Study Methodology

Methodology Development

Study Overview

Scope

A longitudinal clinical cohort study of patients soon to undergo primary total knee arthroplasty were prospectively recruited to a study including the sharing of clinical data, collection of a muscle tissue biopsy, multiple questionnaire completion, and a series of research data collection clinics where functional tests and clinical outcomes assessments were performed.

Study Duration

A maximum follow-up time-point of 12 months post-surgery was set. As many data collection points as reasonably possible were used. Subjects were consented to the study, assessed prior to surgery, had a skeletal muscle biopsy taken during surgery, and then assessed at numerous time-points up until 12 months.

Routine Clinical Context

To aid observational surgical cohort study design, routine procedural steps were established. The patient protocol stages for total knee arthroplasty in the local university hospital are displayed in Table 3.

Variations in this protocol occur if a patient becomes medically unwell. Additionally, during recovery, variation occurs between patients who are referred for physiotherapy intervention in the community, those who self-refer, and those who do not undertake any intervention at all.

Table 3 – Time-points for routine clinical protocol for total knee arthroplasty patient at the Orthopaedic Surgery Department, Royal Infirmary of Edinburgh, NHS Lothian. Protocol correct as of August 2017 at study outset.

Time-point	Patient Activity
Surgery -2 weeks	• Attend outpatient pre-assessment clinic for preoperative baseline health and social checks.
Day of Surgery	• Patient fasting solid food from night before. • Day of surgery health checks at Orthopaedic admissions clinic. • Wait in waiting area until set place on surgical list. • Anaesthetic administration. • **Elective total knee arthroplasty surgical procedure.** • Initial monitored anaesthetic recovery in theatre recovery area. • Transfer to in-patient recovery ward.
Surgery +1 day	• Standing, mobility, and functional status assessments performed by occupational and physical therapy teams.
Surgery +3-5* days	• Discharge from hospital into community once able to perform appropriate activities of daily living independently. **This step varies based on the strength and pain tolerance of the patient, and may be longer if the patient becomes medically unwell.*
Surgery +2 weeks	• Removal of surgical clips by nurse practitioner in the community.
Surgery +6 weeks	• Attend hospital outpatient department for clinical assessment by arthroplasty practitioner or surgical team.
Surgery +12 months	• Attend hospital outpatient department for clinical assessment by arthroplasty practitioner or surgical team.

The clinical protocol identified patient hospital visits. Connecting research data collection with these time-points was beneficial for both the research team and for potential participants, due to a reduced time and travel burden. Major data collection time-points were therefore linked to the routine clinical protocol. However, this did not allow a patient's recovery trajectory to be measured, therefore a sub-cohort of patients was established who could attend extra appointments in the early post-op phase. Creating these time-points for all participants would not have been logistically possible and would have served to dissuade some individuals from participating at all.

Data Collection Time-points

The study time-points were selected to coincide with the routine clinical appointments and to access meaningful and insightful data. Initial post-arthroplasty functional recovery progression occurs at a faster rate (213). The typical functional difference between 1 and 2 months after surgery shows more improvement than the difference between 3 and 6 months, however there is individual variation. Capturing the nuances of this recovery trajectory were important, so more early recovery appointments were chosen, with decreased frequency closer to the final 12-month time-point. The data collection time-points were selected as Pre-Surgery and then at

6 weeks, 12 weeks, 6 months, and 12 months-post-operation. Additionally, two of the post-operative data collection time points coincided with the local NHS protocol at the time. Patients would routinely return to a clinic for assessment by an arthroplasty practitioner at roughly 6 weeks and 12 months after surgery.

The collection of functional data at the surgical pre-assessment clinic (PAC) attendance was not thought to influence physiological data in the surgical biopsy due to the subsequent elapsed time of minimum one, and usually two or three weeks.

Participant Recruitment

As an explorative study to determine relevant factors, no formal power calculation was performed. The recruitment target was determined by surgical list access and the recruitment time window and set at 100 patients within a 4-month window based upon patient availability.

The cohort comprised of patients from the local area who were to undergo primary total knee replacement following diagnosis of end stage osteoarthritis. The annual operating rate at the local hospital site is roughly 700 primary TKA operations per year. This workload is divided between approximately 15 consultant orthopaedic surgeons. While potentially a variable controlling factor, recruiting study participants from the surgical list of only one consultant surgeon would have limited recruitment potential. As such, 11 surgical teams participated in biopsy collection. The nature of the standardised arthroplasty procedure, similar training background of all the surgical teams, and the diagrammatic explanation of requested biopsy location, all served to reduce concern about variation. The target recruitment for the study was set at 100 patients within the time period of August to December 2017.

Participation Criteria

Core criteria were established for the screening of potential participants. They allowed for careful selection of patients to prevent approaching anyone who had any confounding conditions.

Inclusion criteria were to include elective surgery listing, ability to consent, and an agreement to attend follow-up research clinics. The study exclusion criteria were to include comorbid factors that would directly affect function, TKA procedures performed solely for pain relief, and factors limiting data collection such as a lack of fluency in English.

Formalised criteria are tabled in the Methodology section.

Clinical Research during Routine Clinics

To arrange clinic access to approach patients and collect data, setting agreed access times to routine facilities were a key part of the research project. Access is coordinated in the United Kingdom (UK) as Site Specific Approvals with the local hospital facilities. This was organised with the NHS Lothian Research and Development Office who considered the proposed research plan and how it would affect local routine practice.

Permission to conduct a Clinical Research Study

In order to perform research using human participants, ethical review and permission was sought for the proposed protocol. In general, the ethical review process serves to safeguard their rights, dignity, and safety (283,284).

A system exists to streamline the process of applying for Research Ethics Committee and NHS Research and Development study permissions, named the Integrated Research Application System (IRAS) (285) (BGO Software, Sofia, Bulgaria). Once complete, the application forms are automatically generated in PDF format for each authority to review.

Muscle Assessed Knee Replacement Outcome – The MAKRO Study

All local clinical studies are provided an acronym for reference purposes. The study acronym was created and styled as the Muscle Assessed Knee Replacement Outcome study, or MAKRO study. The study was also given a longer study title of ‘Intrinsic muscular physiological factors contributing to early outcome status after primary total knee replacement’.

Ethical approval was granted by South East Scotland REC (REC:17/SS/0088) in July 2017 and NHS Lothian agreed to act as research sponsor (R&D:2017/0189). The study was registered on the NHS Health Research Authority website database (286). The approval documents and the summary study overview can be found in the Clinical Study Approvals, Notices appendix.

Recruitment Documentation Development

The patient information sheet (PIS) was used to inform the patient about what would be required of them to participate in the study. The consent form was used to explicitly state the necessary permissions for when the patient was agreeing to study participation, and to collect their signature.

The PIS content informed the patient about several legal aspects of the study. The confidentiality aspects whereby all identifiable data were subject to the Caldicott Guardian controls (287). That all samples would be eventually discarded in line with the Human Tissue (Scotland) Act 2006. Additionally, it included the details of the study organisers, sponsors, ethical approval details, and contact information regarding requesting any further information or making a complaint.

The consent form explicitly asked for permission for the research study to access their medical records, collect a tissue biopsy sample, for analysis to be performed on the sample, for their GP to be contacted, and for the waiver to any financial gain from participating. They also confirmed that they had read the PIS, considered all the information, and had asked all questions that they had. Lastly, they were aware they could withdraw from the study at any time and without the need to provide a reason.

The PIS was designed with lay accessibility and reading age in mind. The Flesch reading ease score evaluates average word and sentence length to provide a widely used approximation of readability (288). The document attained a Flesch reading ease score of 60.7. The majority body of the text achieved a score above 70, but the necessary inclusion of technical aspects lowered this overall. The scores achieved ensured ease of document comprehension for the patient demographic. The Tegner has a low Flesch reading ease score due to its listicle structure (289). As such, it was discussed with every participant in detail (Appendix A: Clinical Study Documentation).

Patient study data were handled in line with local policies and all legal requirements. Identifiable physical data were stored in locked areas accessible only to study approved personnel on within NHS facilities. Digital identifiable data were located in NHS servers with similar access controls. For analysis purposes, all data leaving these

areas were pseudonymised to allow transport and use with University areas and computers for study purposes.

During the study, the General Data Protection Regulations (EU 2016/679) came into effect via the Data Protection Act 2018. The patient informed consent process as part of study participant enrolment covered all aspects of required data use during the study. Patients were also reminded of this and their rights as research study participants during follow-up clinic visits followed this change in law.

Baseline Participant Data Selection

Patient identifiers and relevant background data were initially collected during initial appointments. These data served to categorise patients demographically and to ascertain background characteristics. Data included are listed in Table 4.

Table 4 - Data collected as part of the study baseline interview with study participants.

Study participant baseline data collection	
Criteria	**Additional information**
Occupation(s)	Sedentary / Labour
Other lower body arthroplasty	Previous lower body joint replacements
Bloods	Haemoglobin, Urea, Potassium, Creatinine, Alkaline Phosphatase, Gamma-glutamyl transpeptidase, Alanine aminotransferase, Bilirubin.
Comorbidities	Discussion following Charlson Comorbidity Index questionnaire
European (Scottish) Tegner Score	Peak fitness, last year, currently
X-ray Kellgren & Lawrence Score	
Dominant side (Left / Right / Ambidextrous)	
Smoking	Current, history, when stopped.
Alcohol	Current, history, when stopped.

Case Report Form Data Selection

The study case report forms (CRFs) were used to record data at each study time-point visit. Multiple patient identifiers were included to confirm participant identity. These were provided linked anonymised identifications for later analysis.

Each CRF recorded time-point biometric information and functional performance data. These were separately obtained from functional assessment instruments and

battery tests. Patient general health state and relevant recent information was also recorded.

Functional Assessment Selection

Several methods for evaluating functional outcome assessment of TKA patients were evaluated. These were based upon OARSI recommendations and local constraints.

Existing pieces of equipment were independently evaluated as to whether or not they would provide valuable insight into the assessment of the patient group. Some pieces of equipment were discounted due to risk assessment conclusions such as weighted tests or tests that may result in a patient fall or stranding. All tests had to be performed within or adjacent to an allocated research space embedded in the Royal Infirmary of Edinburgh's (RIE) orthopaedic outpatient department. While the potential existed to pursue these in other locations, a combination of limitations including the existing available space, support facilities such as waiting areas and administrative staff, and the whether an area was appropriate for the evaluations to take place in were taken into consideration. Additionally, while some clinical research appointments could take place during flexible windows, others were constrained by NHS clinic frameworks. The clinics set appointments to 20 minutes which directly limited the volume of data that could be obtained.

The Aggregated Locomotor Function (ALF) score, and the Nottingham Leg Extensor Power Rig were chosen alongside biometric measurements (290,291). The ALF score provides a composite timed score representing many of the recommended OARSI performance test criteria and utilised department facilities as best possible. The Nottingham Leg Extensor Power Rig directly assessed quadriceps power strength and provided a comparison to body weight (7,124,292,293). All tests are considered valid, reliable and responsive functional outcome assessments within a TKA population.

When studying recovery, a patient appropriate control is used. This can be age and sex matched, however leg strength and functional performance will still significantly vary when this is performed. A potential control to a limb intervention is the contralateral joint. While controlling for many aspects, a reality of the OA TKA

population is frequent presence of bilateral symptoms. The variation and progression of these symptoms can therefore change per assessment time-point and confound results (128,294,295). To prevent these discrepancies, when assessed as non-symptomatic, the contralateral results at baseline can be used as control for the duration of the study, however the bilateral symptoms may still impact this. For example, in a patient awaiting staged arthroplasty on the contralateral knee.

Output data from the Nottingham Leg Extensor Power Rig included raw power generation in Watts and a normalised value controlling for body weight. This controls for sex and stature of subjects (291). Data were recorded for both the operated leg and the control leg at each time-point. They were displayed as a ratio. Both raw output and power-to-body-weight ratio were recorded to provide a range of metrics from the assessment.

Activity Monitoring Device Criteria

Community-measured activity levels were a chosen metric for participant assessment. The selection of an economic and reliable device was necessary.

A sample of ActivPal devices were trialled for this study but frequently detached from their leg placements on departmental volunteers, and reported patchy data when analysed.

The factors of price, availability, features, and reliability were considered while creating a short-list of replacement devices. A wearable device was necessary to reflect the full depth of activity data. On consultation with a patient group, the undesirable connection was made between potential ankle-based research activity monitors and similar devices used by the criminal justice system (296,297). Comments were made that such devices would likely be removed by study participants, resulting in missed data capture. As such, the social context much favoured a watch-like wrist-based activity monitored for protocol adherence.

Factors dictated a device that would be provided to study participants during clinic visits, to be worn for a period of time following the clinic, then returned via postal mail, and synchronised upon return to capture the data from the time period.

Activity Monitor Selection and Data Collection

The Mi Band 2 device (Xiaomi, Beijing, China) demonstrated multi-test reliability of features compared to other devices and possessed a reasonable price point (235). Functions include step count, heart rate, approximations of calorie expenditure, sleep duration and depth, long battery life, and a IP67 waterproof rating (298). It additionally had the flexibility to interact with third party applications in order to divulge raw device data.

Several applications were evaluated for suitability, with the expansion to virtual machine providing greater application flexibility. The VirtualBox desktop hypervisor (Oracle Corp., CA, USA) allowed for emulation of the Androidx86 Marshmallow 6.0 operating system (Google LLC, CA, USA). A bridge was created to Bluetooth hardware to allow device synchronisation to the machines.

The applications Mi Band Master and Notify and Fitness for Mi Band were established as the third-party applications of choice. Both are available on the Google Play application listings. They allowed for fine control of the data capture alongside facilitating the export of raw data via the Dropbox cloud repository (Dropbox Inc., CA, USA) in SQLite database file format. Files are accessed through the DB Browser for SQLite open source software application and exported to comma separated value (CSV) files for analysis. Raw data time-points use Unix timestamps which count the number of seconds elapsed since the start of January 1st, 1970. Having established a working protocol, an additional 9 devices were ordered, each of which had a cloned virtual machine created to unify the device settings and recording instructions.

The duration of data collection was decided based upon the device battery life and the interval between research data collection points. A number of devices had to be ready at all times for data collection, while others may be in use. With daily patient recruitment expected at the start of the study, a reasonable compromise was made

between data collection and device readiness. As such, a period of four whole days was set for data collection. This allowed both for representative data capture and for reasonably prompt device return, especially in time for the latter time-points' weekly research clinics. This duration also reduced the patient burden.

Regarding the collected data an evaluation was made of the hierarchy of data quality. The step count showed strong reliability considering the limitations mentioned earlier. Any periods of waking at night, defined by movement level, were recorded separately. Patient pain and comorbidities may influence sleep data, but are factors which are very relevant to the target investigational cohort.

Reporting of activity tracking data was therefore average daily step count taken from midnight to midnight over 4 days, average resting heart rate between 02:00 and 04:00 over 4 days, and average nightly sleep duration taken over 4 days. The devices collected walking vigorousness, and walking cadence, however there was a lack of clear defining boundaries between device defined categories.

Patient Reported Outcomes Measurements (PROMs) Methodology Development

PROMS Selection

Care must be taken with questionnaire selection as each questionnaire or PROMs tool has been developed for a specific target audience. This includes specific patient groups, population demographics, or specific international classification of disease (ICD) audiences.

PROMs tools are subjected to analysis during development to prevent suggestive scenarios, narrow or confusing answer ranges, floor or ceiling effects, validity, sensitivity, responsiveness, or other non-representative scenarios leading to anomalous data (299). Many PROMs questionnaires provide a numerical output for each dimension or as a total through their scoring rules. The majority of these are scored through two styles of question; numerical rating scales (NRS) and visual analogue scales (VAS). NRS can be binary or Likert scales. Likert scales comprise an odd number of usually ordinal categories as answers to a question. The odd number of categories allows for a central neutral category; however, this is not always used for this purpose. VAS are used to measure subjective attributes that are hard to directly assess. They have continuous line with stated book-end descriptions at the edges where the subject selects a single point on the scale that reflects their answer (300). In hard copy questionnaires, recording of answers is performed by NRS tick boxes and indicating VAS with a drawn vertical line.

Scores are generally cumulative for each PROM dimension and may at times all be combined for a tool total. For each of these cumulative scores, a rubric specific to the tool applies.

While prominently useful in remote data collection, the use of PROM questionnaires allows for time saving within a clinic as they can be filled out by patients in waiting areas or remotely by postal questionnaire. As such hard copy questionnaire batteries were chosen for recording the bulk of this style of clinical outcome data.

Patient questionnaire burden is a factor to be minimised so the dovetailing of existing required departmental questionnaires with study specific batteries was vital (301).

The consideration of burden was also central to selecting the group of questionnaires to be used with the study participants.

The frequently unsupervised nature of PROMs data collection can lead to missing data such as a skipped question. In best practice, patients can be contacted to fill in missed data points, but this is not always possible. Many PROMs tools account in their formulation for this occurrence but it must be acknowledged as a potential factor in the selection of PROMs as a data collection method. For example, in the use of algorithms to retain a comparable outcome score.

During PROM tool selection, all of these criteria must be considered to ensure collected data is reliable and appropriate to the study.

PROMS for Knee Osteoarthritis and Total Knee Arthroplasty

There have been many PROMs tools developed for use the target study population. Considered examples relevant to the study cohort are listed in the Study Methodology Development appendix. The aim was to use a group of questionnaires that would encompass a range of functional, symptomatic, and lifestyle criteria.

A general health and activities of daily living assessment was included as part of the battery. The 36-Item Short Form Survey (SF-36) is a prominently used example of this but takes a relatively long time to complete (302–304). It was established that the Knee Injury and Osteoarthritis Outcome Score (KOOS), Oxford Knee Score (OKS) and EuroQol Five Dimensions Questionnaire (EQ-5D) questionnaires would cover similar ground while retaining a desired knee joint focus in the KOOS and OKS.

MAKRO Study – Chosen PROMs

Having considered the desired data and the nature of each candidate PROMs tool, a final group were chosen to move forward with. PROMs tools selected for the study are listed in Table 5.

Table 5 - PROM tools used within questionnaire groups during the MAKRO study.

PROM tool description	PROM focus	Reference(s)
Charleson Comorbidity Index (CCI)	Comorbidities	(305)
EuroQol Five EuroQol Five Dimensions Questionnaire (EQ-5D)	Health related quality of life index measure	(306,307)
Forgotten Joint Score (FJS)	Joint specific tool	(308)
Knee Injury and Osteoarthritis Outcome Score (KOOS)	Joint specific activities assessment	(309)
Oxford Knee Score (OKS)	Joint specific tool	(310)
Patient Satisfaction (Edinburgh)	Surgical satisfaction	(311)
Tegner Activity Score (TAS) (modified for Scotland based on Scandinavian version)	Work and sporting activities.	(312–314)
Visual Analogue Scale Pain Scores (VAS)	Pain levels	(315)

The CCI was included to cover aspects of relevant comorbidities that may not be overtly present in the patient's notes. A modified version known as 'Complications' was included for post-operative answering to identify any important health issues resulting from or relating to the surgery. For example, a surgical complication leading to a prolonged hospital stay following surgery would influence the physical functional decline (316). Similarly, the presence of pain in a lower limb unrelated to the knee replacement operation would affect functional measurements. This was recorded separately in the Case Report Forms (CRFs).

The EQ-5D, OKS and aspects of the KOOS covered quality of life and ADLs. Knee function was covered by OKS, KOOS, and FJS. Wider activity factors were covered by the KOOS and the Tegner. The chosen tools offered differing and complementary psychometric properties. The whole battery was of reasonable length to be manageable and not a burden as evaluated by an informal consultation with local patient group.

The chosen range of questions covered self-care, acute symptoms, mental health, activities of daily living (such as washing or shopping), recreational activities, surgical satisfaction, and complications. This wide range allowed insight into many relevant aspects of patient recovery.

Tissue Samples and Biopsy Location

Biopsy sampling techniques

The biopsy was taken from the quadriceps muscle group. Both punch biopsy and open biopsy techniques were considered:

Punch muscle biopsies were originally pioneered in 1868 by Duchenne and are taken percutaneously (317). Modern popular muscle punch techniques include the Bergström needle (318) and Tru-Cut needle (319). They are performed using basic sterile considerations and do not require a specialised environment. They allow repeat sampling with relative ease, unlike open biopsies, and carry less risk of infection, however they do require specialist training.

Open biopsies of muscle groups require an incision and surgical operating theatre to provide the necessary infection controls and anaesthesia. This has a greater staffing requirement and cost.

Primary total knee arthroplasty is an open procedure and enabled an open biopsy to be obtained without the need for a separate procedure.

Tissue Optimisation

Due to the expected finite and scarce nature of the proposed study's biopsies, the optimisation of laboratory techniques took place where possible on non-study tissue samples. Other skeletal muscle tissue was collected locally for this purpose through tissue defined as discard material from similar local limb orthopaedic surgeries in an anonymous route akin to internal biobanking (320). This tissue is routinely excised during procedures and is normally destined for clinical waste incineration. Following an informed consent process with the patients prior to surgery, and with the permission of the local consultant surgeons, this tissue could be retrieved. The research was conducted under local ethical permission (LREC number: 2002-1-22; R&D study number: 2002/R/OST/02).

The procedures where the tissue was collected from were anterior cruciate ligament (ACL) repair operations that used hamstring tendon. Hamstring tendon, usually semitendinosis tendon, is harvested and grafted to the native ACL insertion loci (321). The skeletal muscle attached to the tendon at time of harvest is removed and destined for clinical waste incineration in routine practice. With the patients' informed consent, this allowed for tissue collection for research optimisation use.

Local hospital Total Knee Arthroplasty technique and biopsy location

All local consultants and their teams used the medial parapatellar approach to access the knee joint during total knee arthroplasty operations. This approach routinely disrupted and exposed the vastus medialis muscle along the medial border with the quadriceps tendon and the patella. This exposure allowed for collection of the skeletal muscle biopsy without meaningful procedure alteration or an additional procedure. The approach, exposure, and biopsy sample site are shown in Figure 7.

The image was shown to all surgeons involved in the tissue biopsy procedures for study patients. All were given the chance to ask questions and instructed to retrieve a 'pea-sized' muscle biopsy as approved by the ethics committee from the location indicated.

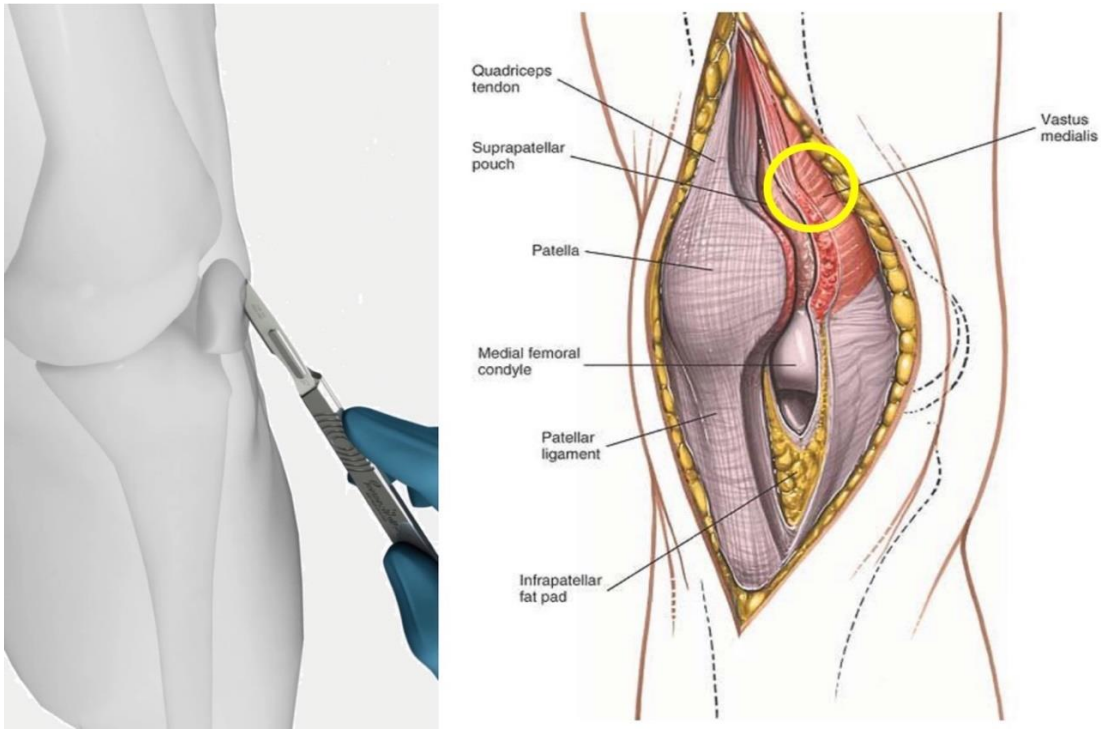

Figure 7 - Peripatellar approach to total knee arthroplasty. Images from Hoppenfeld et al (61)*; Swann Morton.*

The ‘pea sized’ biopsy sample were collected consistently between surgeons. The biopsies were transferred via dry-swab from the surgeon’s scalpel to outside of the operative field, the biopsy was then placed into a sterile universal tube containing woven-gauze slightly dampened with saline. This method was included to prevent drying of the sample (322). Full immersion in saline or other solutions as an alternative would have created large sample artefact from osmotic pressure differences. Immersion in a fixative at this stage would have limited subsequent laboratory processing and analysis, so the decision to keep the samples fresh prior to processing was made. The biopsies were transported back to the research laboratory on an ice pack inside two sealed boxes. This took place within a maximum of 30 minutes from the harvesting of the biopsy.

Methodology

Longitudinal Clinical Cohort Study

Participant Recruitment

Patients fitting study participation criteria (Table 6) were initially approached during their surgical pre-assessment clinic (PAC) hospital appointments. These occurred roughly two weeks prior to surgery. The clinic lists were screened by members of the clinical team for exclusion criteria, leaving a white-list of potential participants. Patients were asked if they would consider participation in a clinical research study and, if amenable, would be provided with a copy of and an explanation of the patient information sheet, and given the opportunity to ask any questions. They were then given the rest of their clinic visit to consider their participation. If happy to participate in the research study, they were provided with a consent form to sign, allocated a study identification number, and provided with the PAC study questionnaire. All aspects were performed by the researcher. Following the clinical aspects of their PAC appointment, baseline physical study measurements were taken. Study documentation are located in the Clinical Study Approvals, Notices, and Documentation appendix.

Not all patients who were listed for primary total knee replacement surgery in the hospital were approached. The main factor in these instances was the dates and times of appointments not coinciding with the researcher's attendance. This occurred due to clinic and surgery, or clinic and laboratory time-conflicts.

Table 6 - Inclusion and exclusion criteria for the MAKRO study at time of study design. Notable is the 5th inclusion criterium which was subsequently adjusted. These were subsequently amended as discussed due to further logistical and clinical considerations.

MAKRO Study Population		
Criteria	**Inclusion Criteria**	**Exclusion Criteria**
1	Adult osteoarthritic patients undergoing primary knee replacement surgery.	Don't meet all inclusion criteria.
2	Willing and able to consent to and comply with the protocol.	Planned bilateral procedures within the study period.
3	Ability to consent.	Procedures done solely for pain relief (e.g. as part of end-of-life pathway).
4	Aged at least 16 years old, there is no upper age limit.	Activity blocking pain in their hip/knee.
5	Ability to attend follow up assessment clinics at RIE site, where they may otherwise attend satellite clinics.	Those not fluent in English.

The location of follow up assessments (inclusion criterium 5) was changed due to repeated requests from potential participants and became reflected in the study sub-cohorts. The second exclusion criterium, of staged bilateral procedures, was strictly adhered to at time of surgery. However, one of the participants had bilateral procedures during the one operation which provided an opportunity to assess bilateral recovery rates. No other participants had planned procedures at time of recruitment, however within 12 months of initial surgery, some were subsequently listed and underwent a contralateral procedure. It would have been unethical and not feasible to prevent this from happening. Their early functional data was still of use, and any subsequently collected PROM data was specified to the study knee.

Study co-enrolment was allowed however no individuals were co-enrolled in research studies during the duration of the study to the knowledge of the researcher, outwith the ongoing local department research database. Full notices and contact details were provided on relevant systems to create alerts if this occurred.

Study Cohorts

Participants were provided with sub-cohort options for taking part in the study. These had different levels of interaction with the researcher during TKA follow-up. The stratification into cohorts was necessary to achieve the maximum possible recruitment in the given timeframe. A trade-off was made between the depth of the functional data and the number of potential biopsies to be collected, and subsequent physiological data. To collect the full data set on all participants would have both been an excessive burden on the research and clinical departments but would also have served to deter participants. This was confirmed by patient feedback.

The study recruitment was initially divided into two cohort participation options. One, named the Routine cohort, would attend their normal routine clinical post-arthroplasty appointments and spend some time during these appointments with the research team. The other, titled Enhanced cohort, would attend these plus two additional post-operative appointments. The Enhanced cohort additionally was the focus of the activity tracker data collection. This cohort was also provided with reasonable travel expenses for the two extra appointments as these were not within

NHS clinic frameworks. The pathways of participation are displayed in Figure 8 which was provided during potential participants' PAC visits.

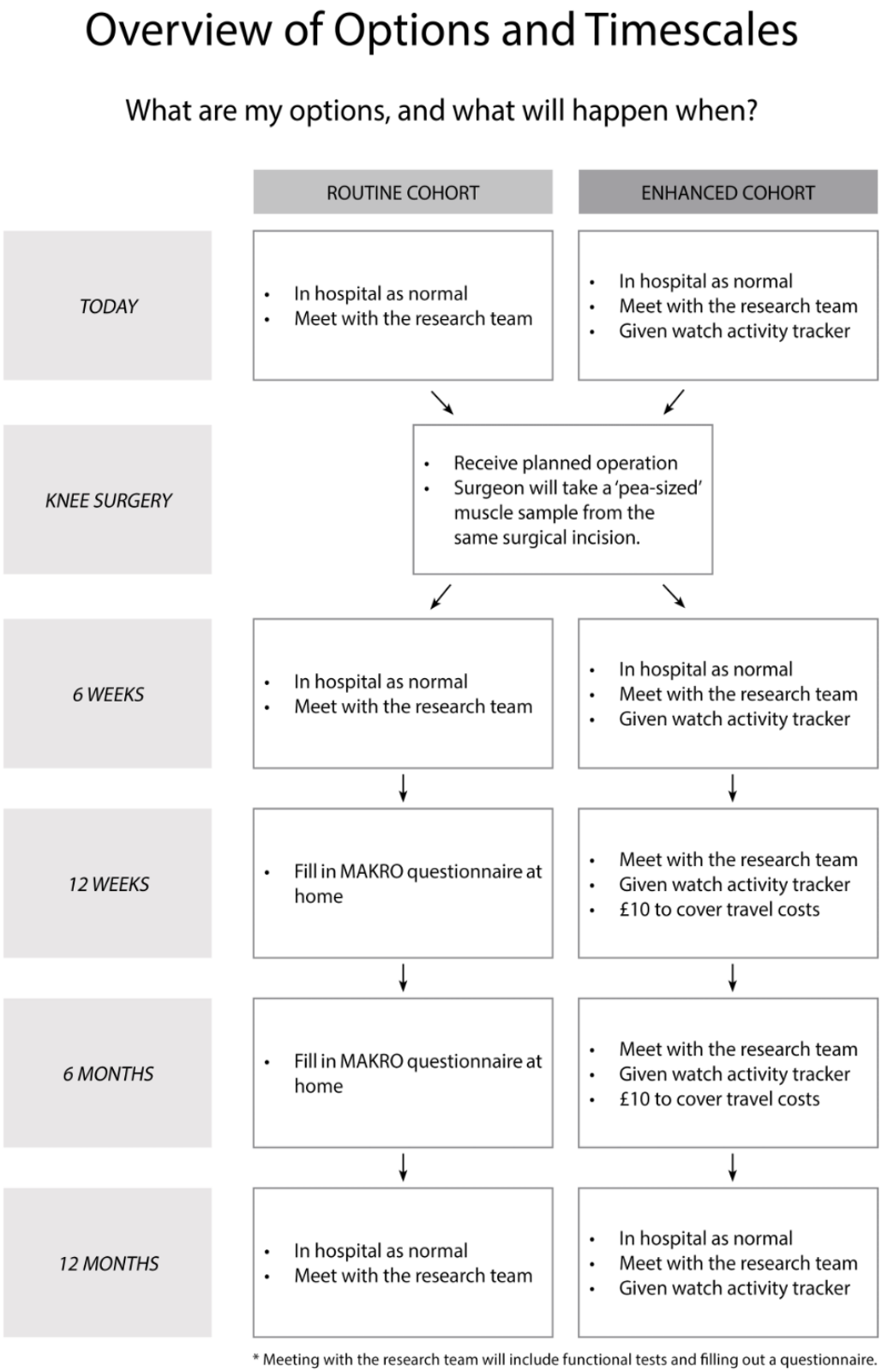

Figure 8 - The MAKRO Study participant cohort pathway options of Routine and Enhanced cohorts with activity details. This was provided to potential participants during their surgical pre-assessment appointments for their consideration as part of the requesting informed consent process.

Latterly, an additional cohort formed where participants could opt to participate in the Routine framework but elect to take their follow-up appointments at regional clinics due to their living circumstances. The nature of the catchment and demographic for the orthopaedic surgery department at the Royal Infirmary of Edinburgh encompassed many patients from the surrounding counties. These patients were glad to take part but felt overly burdened to make a journey lasting for some several hours to attend subsequent appointments. They were still content to provide baseline measurements, provide a biopsy for analysis, and to fill in post-operative postal questionnaires, but were unable to attend for follow-up functional assessments. These participants became known as the West and East Lothian cohort, named after the neighbouring district counties, shortened to the WEL cohort.

The three study cohorts can be summarised as follows. The base level 'WEL' cohort provided base-line measurements, had a muscle sample taken during their knee replacement surgery, and filled out post-op postal PROM based questionnaires. The 'Routine' cohort provided a sample, had functional measurements taken at all routine NHS follow-up time points, and filled in postal questionnaires at the other time-points. Lastly, the 'Enhanced' cohort provided a sample, had functional measurements taken in the clinic at all time points, and were additionally assessed with the activity trackers for four days following each clinic visit.

Study Documentation

The chosen clinical outcome measurements, and patient reported outcome questionnaires were arranged into a machine-readable document format and printed (see MAKRO Study – Chosen PROMs section in Methodology Development).

For the study specific data, in addition to the time-point questionnaire groupings and case report forms (CRFs), baseline measurements of participants were recorded. The document templates can be found in the Clinical Study Approvals, Notices, and Documentation appendix.

Research Clinic Format

The research clinic assessments of study participants were standardised across all time-point. For the initial pre-operative assessment, additional baseline measurement and details were also recorded. These were recorded in Case Report Forms (CRFs) and in the Baseline Report Form for the initial details.

All assessments were conducted in or next to a clinical assessment room designated for research purposes within a hospital outpatient department.

Patient details were recorded by name, date of birth, unique hospital patient identifier (UHPI) number, community health index (CHI) number, along with details about surgery date and operated knee details.

Patient complications, anecdotal functional issues or activity milestones were initially recorded at the start of each clinic assessment. Patient height and weight were then recorded with calibrated NHS Lothian stadiometer and mechanical scales (Seca GmbH, Hamburg, Germany). Patient bioelectrical impendence was recorded with a handheld body composition monitor (Omron BF306, OMRON, Kyoto, Japan) and computed internally via combined algorithms (323–325). Current medication and mobility aid use was also recorded. The range of movement of each knee was recorded with the patient allowed to apply light pressure to manipulate their leg into position. Measurement was made of the passive position that they could comfortably hold their knee at.

Using a Nottingham Leg Extensor Power Rig, patients were asked to perform warm up exercises on the machine to ensure they were comfortable in the assessment position. This encompassed leaning slightly forward in the seat and for the seat to be adjusted to allow full extension of the assessed leg. Maximal extension actions were requested with the instruction given to push as hard and fast as possible. Operated legs were assessed first followed by contralateral legs. Three consistent repetitions were recorded per leg, with reasonable breaks given between them. If a notably lower measurement was recorded, due to a slip or similar, another repetition was measured.

Patients next performed the Aggregated Locomotor Function timed test battery beginning with a timed up-and-go from a seated position containing a walk to a line 2m from the seat, then turn around, walk back, and sit down again. The second part of the test was a timed stair-set ascent and descent. Three steeper stairs were ascended, followed by a 90 degree turn at a landing following by a descent of four shallower stairs. Once down, the timer was reset, and the patient retraced their steps. An average of the two stairs exercises was recorded. Patients were advised to the use the dual bannisters for balancing purposes only, with a test repeat performed if they noticeably swung on them. This was required due to the ALF test validation representative of a "natural comfortable pace" (290). Lastly the patient's eight-meter-walk-test was recorded in an adjacent corridor comprised of a straight line walk to the instruction of at a comfortable pace equivalent to that on the street pavement. The marked distance was slightly longer to allow for acceleration and deceleration.

Finally, average and maximum reported knee pain on a numerical rating scale was recorded. Additionally, at the 6-month post-op time-point, a measurement of knee and thigh circumference was made. This was measured either over tight clothing or against the skin. The patella mid-point marked the knee measure point, and the thigh was measured one third of the distance up the thigh to the hip. All circumference measurements were performed while the patient was standing.

Patient Reported Outcome Measures (PROMs) questionnaire batteries were filled in while in the waiting area or following assessment before leaving. If the time-point and cohort did not coincide with a research clinic visit, these were completed by return-post instead.

Activity monitors were provided to participants of the Enhanced sub-cohort to be worn for 4 days and returned by post in a supplied prepaid padded envelope. Step count, heart rate, and sleep duration and quality were measured by internal sensors.

Patient Reported Outcomes Measurements (PROMs)

The study PROM tools were the EuroQol 5 dimension 3 Likert tool (EQ-5D(-3L)), the Forgotten Joint Score (FJS), the Knee Injury and Osteoarthritis Outcome Score (KOOS), and the Oxford Knee Score (OKS).

The EQ-5D(-3L) questionnaire determined patient generic health status through 5 dimensions resulting in an output index representing quality adjusted life years (QALY). The dimensions are mobility, self-care, usual activities, pain/discomfort, and anxiety/depression. Two visual analogue scales (VAS) representing overall health and pain level are also reported. Index health status values below 0 are termed 'worse-than-dead'.

The Forgotten Joint Score (FJS) comprises 12 questions covering different activities where a patient may or may not be aware of their joint. Forgetting a joint is representative of successful clinical treatment and normality in activity.

The Knee Injury and Osteoarthritis Outcome Score (KOOS) assesses patients' opinion of their knee across 5 dimensions; symptoms, pain, activities of daily living, sports and recreational activities, and quality of life.

The Oxford Knee Score (OKS) examines patients' recent pain and functional experience of their knee.

The PROMs tools were used at each of the study time-points. Each study questionnaire battery was coordinated with the existing departmental questionnaires where required. The existing departmental forms remained unaltered and the study questionnaires were supplementary at those time-points. Departmental PROM questionnaires, for input into the Edinburgh Orthopaedic Research Database, were filled out by patients at their surgical pre-assessment clinics (PAC), and then sent by mail to their home addresses at 6- and 12- months post-surgery. The three questionnaires varied in contents at each time-point. The coordination of each time-point in order to cover all PROMs tools with each repeated

aspect of the PROM battery is highlighted in Appendix A: Clinical Study Documentation. At 6- and 12-weeks after their operations, study participants were only given the study-specific questionnaires.

For the PAC time-point, patients were given both departmental and study-specific questionnaires. Taking roughly 15 minutes to fill out, the hospital visit lasts a few hours and contains many breaks between seeing healthcare professionals when patients remain in a waiting room area. 6-week, 12-week, 6-month, and 12-month post-operative study PROM questionnaires were distributed in person where relevant to those from the cohorts who attended hospital clinic. Otherwise they were sent by post to their home addresses, with a coversheet and a pre-paid and addressed return envelope included. Where necessary, those who failed to return posted questionnaires within a reasonable time-frame were contacted by phone to make sure that it had arrived and that they had not encountered problems with the questions.

The final arrangements of the questionnaires were submitted as part of the study ethical review process. This ensured, alongside estimations of reasonable length to avoid questionnaire fatigue, that the quantity, frequency, and length of study questionnaires would not be excessive.

Once verified by the REC and the R&D Office, the questionnaires were formatted into their final form. A computer form and text recognition program owned by the department was used for this purpose. Formic Fusion (Formic Ltd, Uxbridge, UK) allowed for creation of the grouped questionnaires for each time point as approved. This software package also allowed for scanning and automated input of the completed questionnaires into .CSV format. The formatting process created single documents for each time-point which contained designator barcodes to allow for computer recognition of document item positioning. This allowed the program to identify the time-point and question ID. Scanning of the completed forms allowed for identification of which question box category had been ticked alongside other the information contained in the forms.

PROM Data Processing and Digital Input

All PROMs questionnaires were digitally scanned and inputted into the Formic Fusion software. Questionnaire return-rate varied by cohort, PROM tool, and time-point. The very occasional skipped answers that occurred during questionnaire completion were chased where possible.

After quality checks, the data was exported for processing. Due to the presence of identifiable data, NHS data governance policy dictated that this was removed before transfer from an NHS system. Subjects names, dates of birth, CHI (Community Health Index) numbers, and UHPI (Unique Hospital Patient Identifier) numbers were removed from the data. The remaining identifying characteristic was an allocated study number which was determined based on the individuals order of recruitment to the study. The collected patient reported outcome measure tools were processed and analysed in accordance with their stated algorithms.

Once exported in this manner, data were processed using Microsoft Excel software (Microsoft Corporation, WA, USA). For each PROM tool, the relevant scoring rubric was used. The final score of each tool total or tool subscale allowed for various analyses and comparisons.

Activity Monitoring Data Processing and Digital Input

Activity monitors were synchronised with virtual machines for data capture. Physical devices were sterilised with Distel (Tristel Ltd, Suffolk, UK) and Virkon (Lanxess AG, Cologne, Germany) before synchronisation, charging and reallocation. Raw data was exported and categorised into daily step count, heart rate averages, and sleep duration and depth. Step count data were averaged over 4 days, and sleep data from 3 nights. For ease of analysis, and comparability, sleep depth was calculated as a ratio of minutes of light sleep to minutes of deep sleep.

Tissue Samples

A quadriceps sample was taken at time of surgery from the patient's leg undergoing total knee arthroplasty. Samples were taken from vastus medialis at a single standardised location.

The majority of the samples were transported directly to the laboratory for processing immediately upon retrieval. The simultaneous surgical procedures of some patients, for example for those enrolled in the study who were operated upon at the same time in neighbouring theatres, caused the time window variation. However, all samples were transported to the laboratory within 30 minutes of retrieval, prior to preservation processing.

Chapter 4: Laboratory Methodology

Methodology Development

Sample Processing and Preservation Methodology Development

Samples were to be fresh-frozen and fixed in tandem to provide back-up options. Sample fixation was performed using 4% buffered formaldehyde prior to wax embedding. Multiple options were evaluated for the fresh- or snap-freezing method.

Multiple cooling methods have been used in the literature to freeze preserve muscle tissue for clinical or research purposes (322,326). These include dry ice, liquid nitrogen, isopentane, and other alkane family chemicals.

Freezing through slow cooling or through snap freezing in liquid nitrogen create freeze artefact thereby disrupting histological analysis (327). This occurs due to the slow cooling facilitating local tissue gaseous content expansion, and from local water freezing in a crystalline manner (328). Despite being colder than the isopentane, relatively hot objects immersed in LN_2 are momentarily insulated from the temperature differential because of vapor film formation due to the Liedenfrost effect (329–331). Isopentane thermal conductivity is greater than that of nitrogen and therefore cools a tissue biopsy sample more rapidly (Table 7).

Table 7 - Thermal properties of nitrogen compared to isopentane as candidates for study protocol snap-freezing liquid (332–335).

Material	Melting Point	Boiling Point	Thermal Capacity ($J\ mol^{-1}\ K^{-1}$) (protocol temperature)	Thermal Conductivity ($Wm^{-1}\ K^{-1}$) (protocol temperature)
Nitrogen	-210°C (63K)	-196°C (77K)	70 (77K)	**0.138 (77K)**
Isopentane (2-methylbutane)	-160°C (113K)	28°C (301K)	29 (113K)	**0.171 (113K)**

Cooled propane or hexane were alternative frequently used options for snap-freezing tissue. However, due to their flammable properties, higher freezing point for hexane, and a boiling-point of -42°C for propane, they were excluded during protocol risk assessments.

Snap freezing with isopentane cooled in liquid nitrogen was therefore selected as the snap-freezing sample preservation method.

Histological Methodology Development

Where possible, a number of sections are used to enhance reliability of the histology findings. Variables of the sectioning technique, including temperature, static electricity controls, and section thickness can affect results.

Cryosectioning and Slide Mounting

Samples needed to be cut into sections to undergo histological processing and analysis. Cutting samples into thin sections in this way allowed for visualisation of internal structures and for effective penetrative diffusion of the staining materials. Due to the more versatile nature of the snap frozen samples, these were used for all histological procedures.

Continued preservation of the sample architecture integrity was paramount at this stage. As such, samples needed to be sectioned at low temperature using a cryostat – a microtome within a freezer. A low temperature of -20°C, a 20-degree cut angle, and 8μm thick sections were evaluated as optimal through experimentation with the discard muscle tissue obtained from patients for technique optimisation.

Initial cryostat chuck mounting required careful arrangement to prevent the development of freeze artefact. The use of Optimal Cutting Temperature (OCT) compound (VWR – 361603E) aided this process. OCT allowed for thin sectioning and ease of slide mounting due to the increased surface area compared to the sample alone, reduced section rolling propensity, washed away easily during the staining process, and did not dull the microtome blade. Stored as a colourless liquid at room temperature, OCT became a white solid once cooled below -10˚C which provided its favourable properties. Once set, excess OCT was trimmed with the blade before sample sectioning.

Once cut, the sections were mounted on Superfrost Plus Gold Adhesion slides. The glass slides slides were coated and statically charged to aid their tissue binding properties. Initial optimising run use of basic, non-charged, non-coated slides led to frequent tissue loss during the staining protocols. This necessitated the use of specialised adhesion slides.

Sections were mounted two, three, or four to a slide for each patient sample depending on the destined staining protocol.

Histochemical Staining Optimisation

Initial histological analysis utilised a simple Haemotoxylin and Eosin stain to identify microscopic anatomy for purposes of orientation. Haemotoxylin to stain nucleic acids and eosin to stain protein and highlight cytoplasm. The protocol utilised classic immersion rack and jar staining technique, tap water wash outs, dehydration steps and clearing with histochoice (as an environmentally friendlier alternative to xylene). The full protocol and materials list can be found in the Histology appendix.

The tissue was consistent with the expected anatomy of skeletal muscle and there was a mix of muscle fibre orientations (Figure 9). The biopsy's proximity to tendon interface suggested there may be connective tissue amongst the tissue sample. Samples were visually trimmed of any tissue other than muscle prior to processing, which was confirmed as successful by the histological staining.

Staining to identify sample fibre-types was initially performed through the histological adenosine triphosphatase (ATPase) stain (materials and protocol in Histology appendix). The ATPase stain visualisation functions through the chemical process of cobalt replacing captured calcium in the presence of phosphate. This process is able to differentiate the fibre classes from their defining glycolytic properties resulting in different levels of phosphate and calcium (20,322). The varying use of pH leads to different coloured attributes. Usually, Type 1 slow-twitch skeletal muscle fibres stain light and type 2 fast-twitch fibres stain dark. However, the stain was found to be sufficiently inconsistent in the optimisation tissue that other options

were explored. Examples of successful and poor staining with this method are given in Figure 10. Immunohistochemical stains were found to be more reliable for the purposes of skeletal muscle fibre type identification (336).

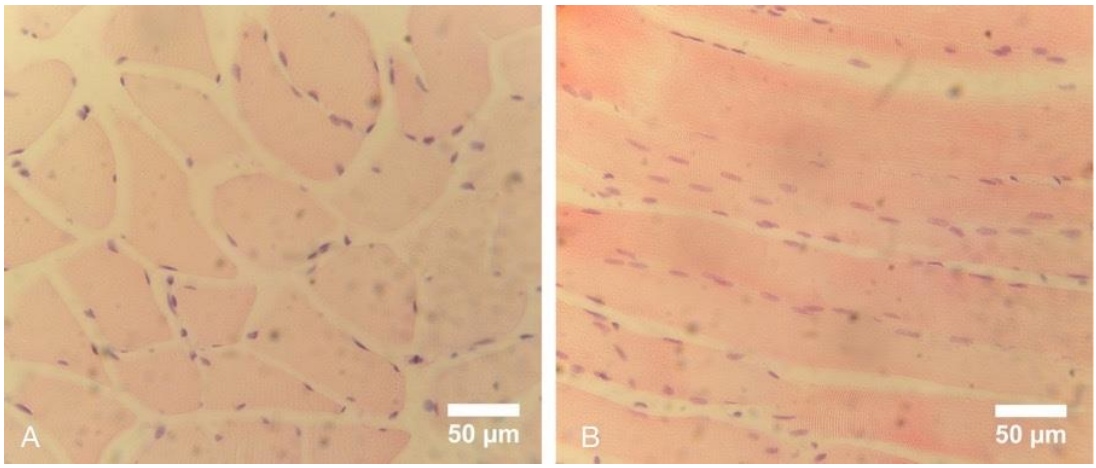

Figure 9 - Haematoxylin and eosin staining of human skeletal muscle tissue during the optimisation process. Some freeze artefact is present. A) Transverse orientation. B) Longitudinal orientation.In order to quantify the desired muscle fibre characteristics and identify structures such as muscle satellite cells, transverse orientation would yield the greatest information. The mixed orientation present amongst the histological sections meant that reorientation was seldom necessary, and with all sections allowing for at least some staining in the transverse plane. To be sure of this, and particularly with the later cryo-sectioning, care was taken to orientate samples transversely when mounting them for sectioning.

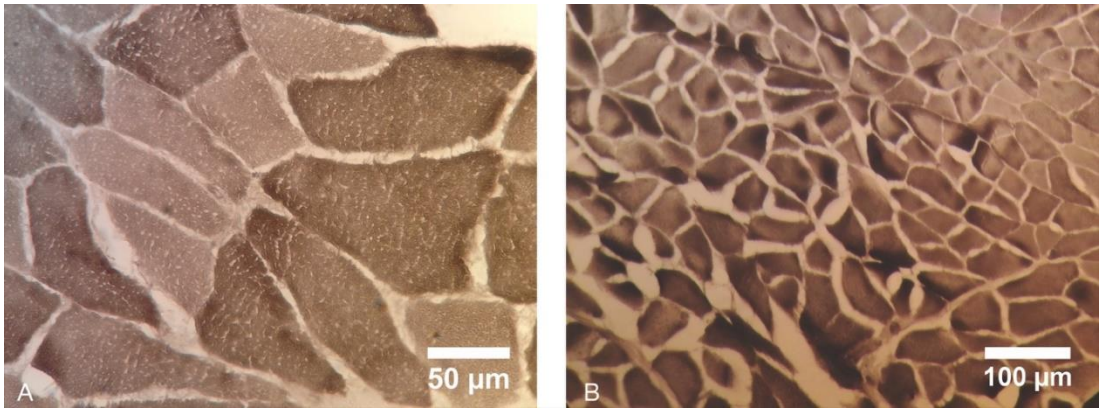

Figure 10 - ATPase histological staining of skeletal muscle tissue. A) Successful staining with type 1 fibres light grey and type 2 fibres dark grey. B) Patchy and inconsistent staining was found to be common despite protocol alterations.

Elimination of freeze artefact

Freeze artefacts were present in the initial histological staining runs. These were defined as the gaps opening up in the honeycomb structure of the natural skeletal muscle (322). They frequently occur due to the expansion of water within the tissue freezing in a crystalline manner, rather than the preferred amorphous freezing which would preserve structure. The expansion of the tissue in this way disrupted the natural tissue form and rendered measured results unreliable.

While the snap freezing process controlled for artefact during the preservation step, more care was required during the mounting step prior to cryo-sectioning. As OCT is stored as a liquid at room temperature, it is much warmer than the cryostat environment when poured onto sample chucks. To allow for dispensing this cannot be avoided. A change in the protocol to introduce careful layering and the use of a fast-cooling cryospray (Cell Path; KNA-0173) provided the needed adaption to remove the freeze artefact (337).

Immunohistochemistry

An overview of immunohistochemistry and immunofluorescence can be found in the Histology appendix.

Identification of Muscle Satellite Cells (MuSCs)

Muscle satellite cells (MuSCs) can be identified through their transcription factors or through combinations of surface membrane proteins (Figure 11). Not all are expressed in all MuSCs at any one time but will vary depending on mitotic state and the subpopulation of MuSCs.

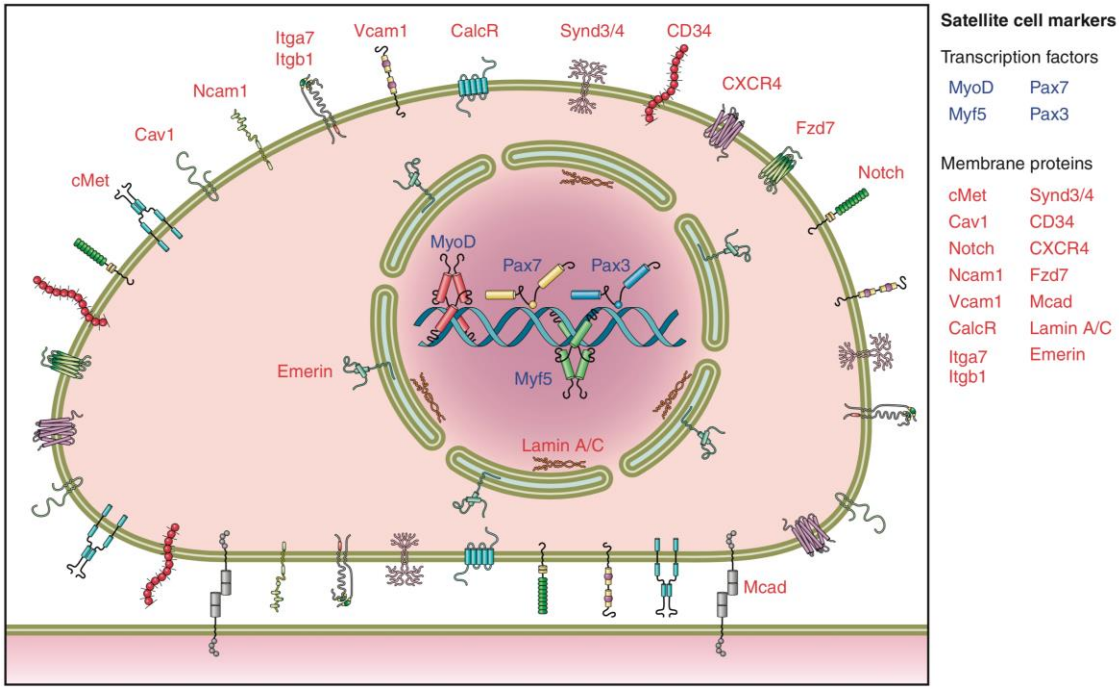

Figure 11 - Muscle satellite cell markers divided by identifying transcription markers and membrane proteins. Not all are expressed in all muscle satellite cells due to population diversity. Figure from Yin 2013 (24).

As described in the literature review section, discussion of the optimal MuSC identifying methodology and associated factors for laboratory analyses are covered in depth in several reviews and study articles (76,172,338–340). Paired box gene 7 (Pax7) and paired box gene 3 (Pax3) are recognised markers for MuSCs along with NCAM1 (also known as CD56). Not all MuSCs express these markers, with some expressing all and some expressing only one. Pax3 is much more prominent embryonically, and NCAM may also identify a subpopulation committed to myoblast differentiation (341).

Muscle fibre types can be histochemically identified through their protein isoform expression as previously discussed (in the Muscle Physiology Research Models and Human Studies section). When identifying major type categories, the labelling of type 1 fibres with MyH7 combined with general morphometry allows for clear distinction between type 1 and type 2 fibres.

Labelling laminin can highlight basal lamina which can allow differentiation between myonuclei and MuSCs. A useful additional anatomical marker is 4',6-diamidino-2-phenylindole (DAPI) which labels nuclei.

The anatomical target structures for the study were muscle satellite cells and muscle fibres. The target antigens for these were selected as anti-paired box gene 7 (α-Pax7) and anti-myosin heavy chain 7 (α-MyH7). As a nuclear stain, Pax7 required a slightly different protocol than the surface membrane marker MyH7.

Immunofluorescence Methodology Development

The laboratory protocols for the IF staining were based upon a variety of published protocols (46,169,340,342–348). Rigorous optimisation runs with control skeletal muscle tissue were performed for all staining panels to ensure optimal conditions were established.

For each staining panel, a variety of methods were trialled. Different fixatives, incubation temperatures, incubation times, and antibody concentrations were

examined to optimise the techniques. Formaldehyde based fixatives created inconsistent staining patterns which were attributed to protein cross-linking. Subsequent antigen retrieval techniques were non-effective. As fixatives, ethanol was too gentle, and acetone was too harsh, in addition to causing damage to laboratory staining apparatus.

Core concentrations were established using published protocols, manufacturer recommendations, and a series of dilutions. The lowest possible concentration of antibody was used to preserve stocks and to prevent tissue artefact, particularly from secondary fluorophores.

Incubations times and temperatures ranging from 1 hour at +37°C, to 3 hours at room temperature, to overnight at +4°C in a refrigerator were tested. Any longer or shorter at each of these temperatures provided insufficient or excess staining. Though a shorter protocol, primary antibody incubations at higher temperatures led to high background staining. Secondary antibodies were unaffected by this.

Muscle Satellite Cell Immunofluorescence Panel Development

To visualise muscle satellite cells (MuSCs) within sections, a combination immunofluorescence (IF) staining panel was to be used. The panel stained for nuclei (DAPI counterstain), MuSCs (α-Pax7), and basal lamina (Laminin 5 alpha-3). This allowed for visualisation of MuSCs nuclei compared to assumed myonuclei, with a confirmatory reference of the basal lamina to differentiate MuSCs based on their differing anatomical location to myonuclei. Initial IF runs also investigated CD56 as a MuSC marker but were unsuccessful. A latter panel combining MuSC and muscle fibre type staining was also evaluated.

Considering antibody selection, factors encompassed primary host species, secondary host species, and fluorophore. Suggestions were made in published research journal articles, but these were not always available from the suppliers.

The list of utilised IF and IHC antibodies for the MuSC panels are listed in Table 8.

Table 8 - List of antibodies used for the Muscle Satellite Cells immunostaining protocols

Antibody Target	Anatomical target	Host	Brand	Product code	Type: α^{I}, α^{II}, conjugated, counterstain	Fluoro-phore (if applicable)	Final con-centration used
CD56	MuSCs	Mouse	BD	347740	Primary	n/a	
Pax7	MuSCs	Rabbit	Bioss	bs-2413R-FITC	Conjugated	FITC	
Pax7	MuSCs	Mouse	DSHB	Pax7-s	Primary	n/a	1:6
Pax7	MuSCs	Mouse	DSHB	Pax7-L	Conjugated	FITC	
LAM	Basal Lamina	Rabbit	Bioss	bs-1969R	Primary	n/a	
LAM	Basal Lamina	Rabbit	Bioss	bs-1969R-Cy5	Conjugated	Cy5	
Primary	Donkey-anti-mouse	Donkey	Fischer-Sci	PA128625	Secondary	TRITC	1:500
Primary	Donkey-anti-mouse	Donkey	Santa Cruz	sc-2300	Secondary	TRITC	
Primary	Goat-anti-rabbit	Goat	Bioss	bs-0295G-HRP	Secondary	[IHC]	
Primary	Goat-anti-rabbit	Goat	Bioss	bs-0295G-FITC	Secondary	FITC	
Primary	Goat-anti-rabbit	Goat	Bioss	bs-0295G-Cy5	Secondary	Cy5	
DNA	Nuclei	n/a	Vector labs	H-1200	Counterstain	DAPI	Stock

Initial runs identified a lack of antibody reliability which did not equate to the success found in the literature. Antigen retrieval techniques, such as Heat Induced Antigen Retreival (HIAR) were utilised to enhance epitope prominence to improve staining (349,350), however this is not routinely described for frozen muscle sections (351). The use of HIAR frequently disrupted mounted tissue sections but was utilised with the aim of improving immunostaining.

Slide mounted sections were post-fixed in methanol at -20°C for 6 minutes. Heat Induced Antigen Retrieval (HIAR) at 100°C took place in antigen retrieval buffer (Vector 3300; 1:10) for 10 minutes within a microwave and pressure cooker (Nordic Ware Tender Cooker). Sections were ringed with a hydrophobic 'Pap' pen (Vector ImmEdge) and washed three times with phosphate buffered saline with 0.05% Tween20 (PBST) for 3 minutes. Sections were protein blocked (DAKO Protein Block x090930) for 15 minutes at room temperature to prevent non-specific antibody binding to the tissue. Incubation of primary antibody mouse-anti-Pax7 (1:6; DAKO Ab Diluent s202230) took place at +4°C overnight. Slide trays included damp paper towels during incubation periods to prevent slide drying. Following this, sections were washed three times with PBST, blocked for 15 minutes, and incubated with the secondary antibody donkey-anti-mouse TRITC (1:500 ; DAKO Ab Diluent s202230) for 1 hour at +37°C. Slides were washed three times with PBST, once with PBS, and then mounted with Vectashield with DAPI (H-1200) and coverslips were placed on the slides. These were sealed at the edges with colourless, clear nail polish and dried before imaging. The full protocol and materials list can be found in the Histology appendix.

Microscopy imaging of the slides followed, and subsequent image processing initially indicated positive and distinctive staining for Pax7 and therefore MuSCs.

Additionally, a separate protocol expansion was created that also incorporated a fluorescence triple stain for Pax7, Myh7, and DAPI. It would identify MuSC fibre type affiliation. The imaging results from the protocol replicated those displayed in the literature for similar panels. A raw merged and a merged channel image adjusted for channel brightness from the protocol are displayed in Figure 12.

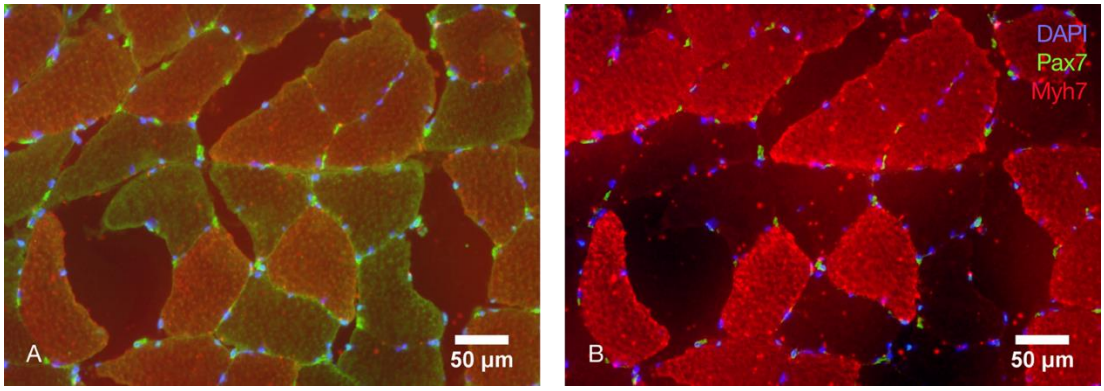

Figure 12 - Images of immunofluorescence staining for DAPI (blue), Pax7 (TRITC – red), and Myh7 (FITC – green). Both images are of the same section with different levels of digital processing. A) Raw image colour channel merge. B) Image channel merge adjusted for brightness. Positive muscle satellite cells are located at muscle fibre periphery and positively stained for both DAPI and Myh7. Though convincing, the FITC expression contained a large amount of autofluorescence which raised questions as to the reliability of the staining.

Instances of colocalised expression between DAPI and Pax7 were prominent and the protocol was initially deemed successful. However, further investigation and confirmatory controls determined that it was likely a false positive and was prejudiced by confirmation bias (categorising visually borderline results as fitting the predetermined positive category).

Auto-fluorescing of skeletal muscle section negative controls was observed under certain fluorescence microscopic wavelengths; primarily FITC, but also TRITC. This contravened published research where frequent use was made of FITC, Alexa Fluor 488, Green Fluorescent Protein (GFP), and TRITC fluorophore tags for small anatomical targets in skeletal muscle (352). The phenomenon is well documented within the cardiac muscle literature (353) but not abundantly so in the skeletal muscle literature. For example, the utilised secondary wavelengths were extensively utilised in the published literature on which the protocols were based. The autofluorescence is most present within these wavelengths.

Despite the existence of autofluorescence in these channels, with strongly expressed targets and high antibody concentrations, identification of anatomical landmarks was still possible. However, this was not the case with low expression of targets and could have provided false positives.

The autofluorescence was likely to be due to the innate presence of flavins and flavoenzymes in skeletal muscle tissue (354). Endogenous autofluorescence was hard to control for, with the exception of differentiating intensity of fluorescence, however distinct spot artefacts were also found during imaging. While each planned immunofluorescence staining run included negative controls, these were for the primary and secondary antibodies respectively. Due to the identification of this artefact and the presence of discussion surrounding the topic in the literature (355), in depth control panels were run for all aspects of the proposed protocol to minimise autofluorescence (Table 9).

Table 9 - Investigative controls for autofluorescence artefact in the Pax7 immunofluorescence staining protocol.

Slide ID	Protein Block	Incubation with diluent	Mount with DAPI	Mount with DPX
A	X	X	X	
B	X	X		X
C	X		X	
D	X			X
E		X	X	
F		X		X
G			X	
H				X

From these investigative runs to identify and potentially eliminate the autofluorescence in the FITC channel, there unfortunately was no clear candidate for the artefact. Though slightly higher background autofluorescence was found in the Pax7/FITC-antibody-tagged IF runs, it also existed in all of the control runs. Examples of the extremes of control from images of the full Pax7 runs, to control slide A (Table 9), and control slide H are shown in Figure 13.

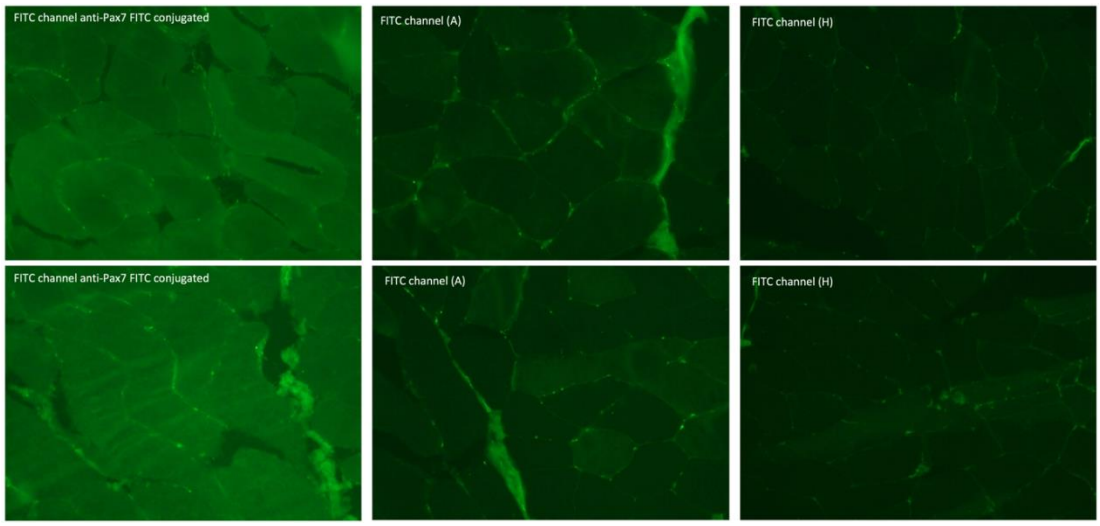

Figure 13 - Investigative controls for autofluorescence artefact in the Pax7 immunofluorescence staining example images. Autofluorescence is present in all images. Of note is the bright spot autofluorescence from the basement membranes and perimysium in the borders between muscle fibres.

Camera exposure or brightness variation may have played a part in the distinct visual differences. The imaging apparatus was automated in this element and self-adjusted based on its internal software algorithms. Standardisation of these elements was not possible.

The Pax7 IF staining protocol was further interrogated. True IF expression labelling was assumed to be inherently brighter than autofluorescence. A manual method to differentiated between the autofluorescence and the positive staining for Pax7 with the image processing software ImageJ (NIH, USA) was created. Colour channel isolation was used to separate and distinguish the intensity of fluorescence within candidate anatomical locations (Figure 14). Each distinct channel was separately processed using ImageJ. This allowed reasonable identification of positive staining for presence of Pax7. Examples images of the method compared to negative control slides are shown in Figure 15. Image autofluorescence across both FITC and TRITC channels was compared for fluorescence point intensity. Distinct superior brightness in the FITC channel and colocalising with DAPI fluorescence was identified as positive staining for Pax7. White arrows represent positive staining for both Pax7 and DAPI. Green arrows represent eliminated false positives.

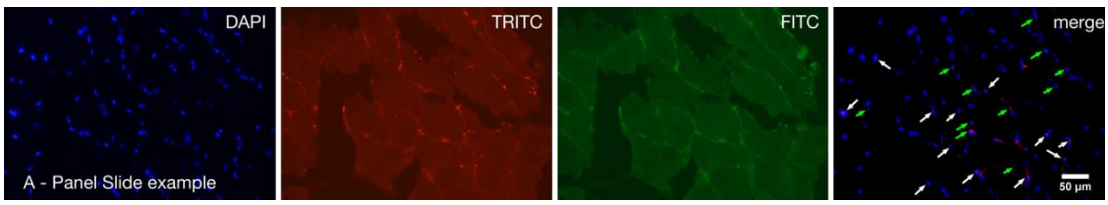

Figure 14 – Colour channel isolation and merging of a single field of view example for manual identification and differentiation between positive and false positive staining.

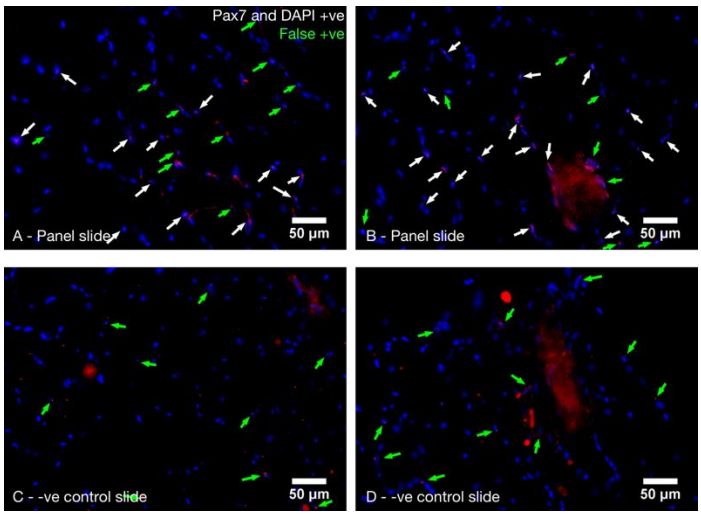

Figure 15 - Examples of the Pax7 immunofluorescence staining panels and connected negative control slides. White arrows show colocalised DAPI and TRITC (Pax7) positive staining. Green arrows show eliminated false positives attributed to autofluorescence. Images A and B show Panel slides (immunofluorescently stained for DAPI and TRITC anti-Pax7), images C and D show negative control slides, with only DAPI staining.

The differentiating image sorting method was reproducible but identified a high rate of nuclei belonging to MuSCs. The method was not standard and accepted image analysis technique. Due to the challengeable aspect of its subjectivity, borderline nature of many of its classifications, the large time burden per image, it was forfeited as a image processing technique.

The utilisation of alternative fluorophores, careful HIAR, and microscopes with a wider range of mirror cubes for imaging may allow for clearer identification of MuSCs in future.

Despite multiple protocols, antibodies, reagents, and creative image analysis to optimise the staining, no reliable protocol was identified to label and identify MuSCs immuno-fluorescently. Alternative methods were subsequently pursued to identify the MuSCs within the study biopsy samples, notably molecular gene expression analysis. However, not all initial hypotheses could be tested with these alternative investigative methods. Namely the local anatomical associations of the MuSCs such as fibre type affiliation and proximity to vasculature.

The study's findings during technique optimisation raise serious questions about the validity of published literature using this Pax7 MuSC immunolabelling and imaging methodology due to the false positives resulting from multiple channel auto-fluorescence in skeletal muscle tissue.

Muscle Heavy Chain 7 Immunofluorescence Panel Development

An immunofluorescence (IF) histological staining panel was developed to identify skeletal muscle fibre type within sections. This utilised IF targeting of Myosin Heavy Chain 7 (MyH7), a gene coding for the β-myosin heavy chain protein. This protein is a component of both cardiac and type-1 skeletal muscle. Given the location of the biopsies, this targeting allowed for identification of type-1 fibres within skeletal muscle tissue. The list of utilised IF antibodies for the MyH7 immunofluorescence panel are details in Table 10.

Table 10 - List of antibodies used for the Muscle Heavy Chain 7 immunostaining protocols.

Antibody Target	Anatomical target	Host	Brand	Product code	Type: α^{I}, α^{II}, conjugated, counterstain	Fluoro-phore (if applic.)	Successful protocol with study tissue? (*)	Final conc-entration used
MyH7	Type 1 muscle fibres	Mouse	DSHB	A4.951-s	Primary	n/a		
MyH7	Type 1 muscle fibres	Mouse	Santa Cruz	SC53090	Primary	n/a	*	1:500
Primary	Donkey-anti-mouse	Donkey	Fischer-Sci	PA128625	Secondary	TRITC	*	1:750
Primary	Donkey-anti-mouse	Donkey	Santa Cruz	sc-2300	Secondary	TRITC		
DNA	Nuclei	n/a	Vector labs	H-1200	Counterstain	DAPI	*	Stock

Reliable primary and secondary antibodies were identified within a viable staining protocol detailed in the Methodology section.

Imaging Microscopy Optimisation

A Nikon E800 fluorescent microscope (Nikon Corp., Tokyo, Japan) was used to image all slides. The microscope was set-up to perform light and fluorescence microscopy (356,357), and contained fluorescence filter cubes for the following fluorophore wavelengths: cyanine 5 (Cy5), 4',6-diamidino-2-phenylindole (DAPI), tetra-methyl-rhodamine (TRITC), and fluorescein (FITC). Excitation and emission wavelengths for these dyes are listed in Table 11 and in Figure 16.

Mirror cube excitation and emission spectra possessed sufficiently distinct ranges to prevent leakage across channels.

Table 11 – Study fluorescent dye excitation and emission wavelengths.(358,359)

Dye Name	Excitation Maximum Wavelength (nm)	Emission Maximum Wavelength (nm)	Visual Colour Range
Cy5	646	664	Far-Red
DAPI	358	461	Blue
TRITC	557	576	Yellow/Orange
FITC	495	519	Green

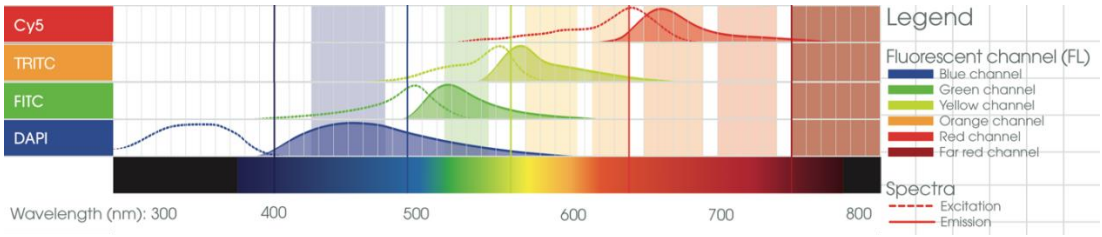

Figure 16 - Study fluorophore excitation and emission wavelengths. Adapted from Abcam Fluorochrome Chart (359).

Optical imaging was performed with a 10X eyepiece with 10X and 20X objective lenses. Working magnification was therefore 100X and 200X. This provided sufficient detail for visualising target anatomical structures.

Digital Image Processing Optimisation

Digital image processing was to be conducted within ImageJ software (NIH, USA). Muscle fibre type and muscle fibre lesser diameter are identified from Myh7 IF staining. Raw microscopy images were divided by fluorescence channel and varied in brightness. This utilised the 'split/merge channels' and 'adjust' functions to normalise each channel and to combine them into single images to be analysed.

Image Analysis

The analysis of the images was performed using ImageJ functions. Initial scale calibrations were set from ingrained metadata that was digitally etched by the imaging camera system. Predominantly, the 'measure' and 'multi-point' tools were used to record the desired measurements. This allowed for accurate measurement of anatomical structures.

For some desired analyses within ImageJ, automated functions exist to process large numbers of images at once. These functions can perform complex image processing and analyses, and ultimately are very time-saving for researchers.

(A plugin macro named MuscleJ has been developed which could have allowed for this to happen with the study image analysis (360). While still in beta development, the authoring team from Institute Pasteur (Paris, France) directly shared the unpublished macro for use with this study image data set. Despite local attempts to optimise the programming elements, the beta plugin was found to have a high error rate when used on the study data set. The plug-in undoubtedly has promising potential for future use in anticipated newer versions. All images for the data set were therefore manually processed.)

Manual digital image processing and analysis allowed for clear labelling of distinct fibre and their diameters. Examples of the fibre classification analysis are displayed in Figure 17. The labels '1' and '2' refer to the skeletal muscle fibre types.

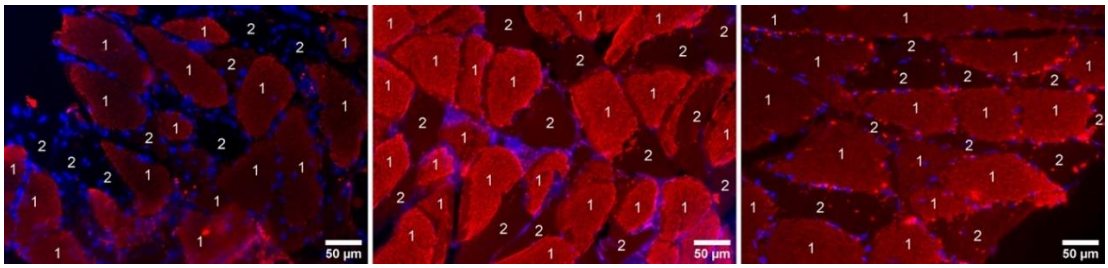

Figure 17 - Examples of the analysis method on ImageJ software for fibre type categorising. '1' represents Type 1 skeletal muscle fibres. '2' represents Type 2 skeletal muscle fibres.

Confirmatory Analysis Techniques

Further laboratory techniques were explored to provide a larger depth of data than that from the histological analyses alone. The methods sought to include quantified sub-population cell counts for entire biopsies, rather than representative sections.

Flow cytometry and quantitative real-time polymerase chain reaction analyses were evaluated due to their prominence in the literature (339,361).

A representative frozen biopsy sample stored at -80°C for four months was chosen to evaluate a preliminary flow cytometry protocol based on a published MuSCs isolation publication (362). Investigations of the biopsy cell flow viability was performed at the Shared University Research Facilities' (SuRF) with propidium iodide to establish a "live/dead" count (363). Out of 80,000 events, 289 whole cells were gated (0.4% of total), of which 271 cells were alive (93.8% of whole cells; 0.3% of total). The full protocol and materials list can be found in the Cytometry appendix. This low percentage of intact cells indicated that the digestion methodology was unsuitable for the study biopsies. The biopsy collection and storage procedure, including ultra-low temperature storage, may also have influenced the whole cell integrity. While whole cell analysis was not appropriate, the tissues were suited to alternative genetically based analyses as molecular nucleic acids were unaffected in this way.

Quantitative real-time polymerase chain reaction (qPCR) analysis was utilised to examine quantified whole-biopsy genetic expression profile for the characteristics of interest.

Genetic Expression Analysis Methodology

Quantitative real-time polymerase chain reaction (qPCR) allowed investigation of myogenic marker profiles within patient biopsies. These would provide data on the genetic expression levels of target markers at time of surgery.

Previous departmental work had investigated similar markers within an oncological population using quantitative real-time polymerase chain reaction analysis (qPCR) (137). Applying this technique to analyse the TKA cohort biopsy samples allowed for the quantification of target markers of both myogenesis and senescence as discussed in the literature review.

RNA Extraction Method Choice

Methodology options for RNA extraction included commercial kits and a Trizol method. The RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) was compared to a Trizol extraction method (364–366). The full protocols and materials list can be found in the Molecular appendix.

Initial results from the kit indicated contamination and poor extraction. To determine whether the results were reflective of cohort sample quality or due to the extraction method, extraction was run in parallel with two cohort samples using the two extraction methods. Each patient sample was split equally in two, with one half of each sample per method.

Results from the tandem run showed a much higher purity and yield from the Trizol method compared to the Qiagen kit (Table 12). This was measured using the NanoDrop ND-1000 machine (NanoDrop Technologies Inc., DE, USA), protocol available in the RNA Analysis appendix. The two yield ratios provided information on the purity of the extract. The 260/280 and 260/230 ratios compared the ultraviolet light absorbance of the samples at the specified wavelengths in nanometres. For extracted RNA, desired purity ratios values are ~2.00. This optimal number were found from the averaging of the absorbance values of all four nucleotides. Each nucleotide taken separately has differing values, from guanine providing a ratio of 1.15 to adenine's ratio of 4.50. Depending on which bases were present, the true

ratio can be affected. The values can additionally vary by 0.2 from this due to differences in pH, with acidity reducing the value and alkalinity increasing the value (367). A low value of the 260/280 ratio can indicate contamination of the sample with protein. When seen in the 260/230 ratio, it can be due to the presence of phenol or ethanol. Contamination affects the values due to contaminants strong absorption properties at the focus wavelengths (368).

Table 12 - Comparison of purity and yield data between extraction methods.

Sample	Extraction method	260/280	260/230	RNA yield (ng/μl)
A	Trizol	1.91	1.97	475.6
A	Qiagen kit	1.88	0.75	64.5
B	Trizol	1.90	1.34	213.5
B	Qiagen kit	1.50	0.44	20.0

The Trizol method was therefore chosen for the cohort's skeletal muscle biopsy RNA extraction due to the larger yield and purity potential.

Trizol is a monophasic solution of phenol and guanidine isothiocyanate that disrupts cells and dissolves cell components while maintaining RNA integrity. The steps include the lysing of cells, separation of cellular material into distinct phases, the precipitation of RNA from an isolated component of this, and finally the washing of the isolated RNA pellet to remove contaminants.

RNA Quality Analysis

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines state that RNA quality must be stated in experiment methodology, and further quality assessments are desirable. The quality and purity was to be assessed by the NanoDrop spectrophotometer, and integrity was next investigated (369).

RNA integrity represents the quantity of the extracted total RNA that is intact. It is measured through a calculated ratio between two molecularly weighted peaks of ribosomal RNA (rRNA) identified through electrophoresis; 28S and 18S (370). 28S represents part of the large subunit and 18S represents the small subunit. Electrophoresis uses a porous gel and an electric current to separate electrically

charged nucleic acids based on size. For intact total RNA, clear and distinct bands should be observed at these locations following electrophoresis analysis. With fragmented RNA, the result is dispersed and indistinct. Locations, based on size differences, can be identified through the use of a molecularly weighted control ladder. While a qualitative indication of RNA integrity can be evaluated using gel electrophoresis, quantitative data can be established using instrumentation. Automated electrophoresis can calculate the quantitative RNA Integrity Number (RIN) from algorithmic analysis of the generated data (371).

The TapeStation 2200 (Agilent Technologies Inc., CA, USA) was used to provide automated electrophoresis and analysis of extracted sample RNA for this purpose. The machine set protocol was followed. The full protocol and materials list can be found in the RNA Analysis appendix.

A selection of optimisation samples were analysed for RIN with results showing inconclusive values (Figure 18). This was due to control ladder values also were inconclusive indicating a machine or consumables malfunction. The ScreenTape machine consumables are all-in-one reagent chips in which all samples are run by electrophoresis. While repeat freeze-thaw cycles can increase the degradation of RNA (372), if this was the error source the controls and ladders would have had positive results. High integrity can be identified from values close to 10, however the originating tissue type is known to lead to reduced RIN when assessing extracted RNA (370). However, the machine fault across the preliminary experimental runs indicated an issue with the chips.

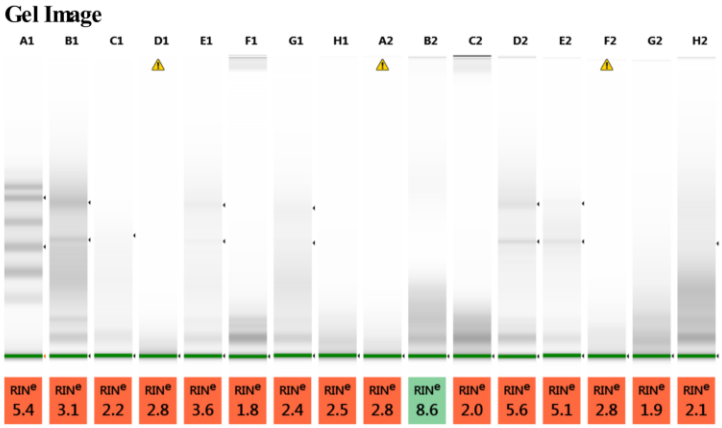

Figure 18 - Tapestation preliminary experimental results showing machine malfunction. The ladder (far left) should shown clear banding with a high RIN value.

In line with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, the Nanodrop quality and purity analyses were sufficient for identifying nucleic acid properties for study samples during initial extractions.

Real-Time Polymerase Chain Reaction Optimisation

The quantitative real-time polymerase chain reaction technique allows for target genes of unknown expression levels to be compared against consistent and relatively highly expressed known reference genes in a quantitative manner. The reference or housekeeping genes were first identified using representative samples from the cohort. They were identified by performing optimisation qPCR runs using commercial kits of likely gene candidates that were known to show stable high express. From these, an algorithm can select the best combination of genes that were stable in the cohort tissues.

The comparison between the target experimental genes and the reference genes was performed by comparing cycle threshold (C_t) or quantitative cycle (Cq) values. These represent the PCR cycle at which the detected quantity of PCR product cross a pre-set level on the thermal cycler machine. The quantity was calculated by the fluorophore expression strength, which is replicated, and in-theory doubled, per cycle. The amplification past the C_q at an earlier cycle represents a higher starting quantity of the gene of interest (GOI), and therefore a higher expression of that GOI in the sample. As all mastermixes, the starting content in each well in the instrument, are identical in volume, the expression levels can be compared. For each sample, the resulting comparative cycle threshold difference (ΔC_q) between the experimental GOIs and the reference GOIs values were then able to be quantitatively calculated. The ΔC_q values were then able to be compared between genes, and subsequently to other patient cohort factors.

Reference Gene Selection

The quantitative value of qPCR lies in a normalisation procedure. Genes of interest (GOIs) expression levels are measured relevant to reference or housekeeping genes. As such, the initial step required justification for the normalisation procedure and

identification of which reference genes were best for the tissue. The perfect reference genes should be consistently expressed across all the samples with high expression stability. The MIQE guidelines recommend using more than one reference gene, with a geometric mean of those used to normalise the GOI fold expression.

To determine the best reference genes, a panel of 12 common qPCR reference targets (Human geNorm kit; ge-SY-12-hu; Primerdesign Ltd, Southampton, UK) were analysed. The reference gene candidates are listed in Table 13.

Table 13 – The 12 qPCR reference gene candidates.

Gene Name	Gene Function (373)
SDHA **Succinate dehydrogenase complex flavoprotein subunit A.**	Encodes major catalytic subunit of the mitochondrial respiratory chain.
CYC1 **Cytochrome c1**	Role in cell proliferation.
18S **18S ribosomal RNA**	Eukaryotic cytoplasmic ribosomal subunit.
TOP1 **DNA topoisomerase 1**	Encodes a DNA topoisomerase, an enzyme that controls and alters the orientation of DNA during transcription.
ATP5F1B **ATP synthase F1 subunit beta**	Encodes a subunit of mitochondrial ATP synthase.
UBC **Ubiquitin C**	Encodes a polyubiquitin precursor.
GAPDH **Glyceraldehyde-3-phosphate dehydrogenase**	Product catalyses a step during carbohydrate metabolism, also has uracil DNA glycosylase activity in the nucleus, and contains peptide involved in antimicrobial activity.
B2M **Beta-2-microglobulin**	Encodes a serum protein found on the surface of most nucleated cells.
ACTB **Actin beta**	Encodes one of six different actin proteins, involved in cell motility, integrity, structure, and intercellular signalling.
RPL13A **Ribosomal protein L13a**	Eukaryotic cytoplasmic ribosomal subunit.
EIF4A2 **Eukaryotic translation initiation factor 4A2**	Regulates lipid metabolism, and translation factors.
YWHAZ **Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta**	Encodes mediator of signal transduction.

A representative selection of 12 study samples were used to evaluate the reference gene candidates. These represented a quarter of samples and were chosen based upon widely representative criteria to encompass several participant sub-cohorts. These included: sex, BMI, age, arthritic severity, and lifestyle choices. All samples were run in triplicate against all reference primers.

The supplied kit protocol was followed. The qPCR master mix was created for all genes and plated into a 96-well plate before the addition of each chosen sample. Pipette tips were changed for every triplicate to remove potential carry-over. The qPCR program was run on the LightCycler 96 Instrument (F Hoffman-La Roche AG, Basel, Switzerland) with the protocol displayed in Table 14 and Figure 19 - Graphic representation of qPCR protocol for the geNorm reference gene data collection.. Fluorometric data was collected through the detection of the intercalated dye SYBR Green, with greater fluorescence indicating greater presence of qPCR product(350). The full protocol and materials list can be found in Appendix B: Laboratory Protocols.

Table 14 - qPCR protocol for the geNorm reference gene data collection.

Step	Temperature (°C)	Duration (seconds)	Number of Cycles
Preincubation	95	120	1
Amplification	95	5	45
	60	20	
Melting	95	10	1
	65	60	
	97	1	
Cooling	37	30	1

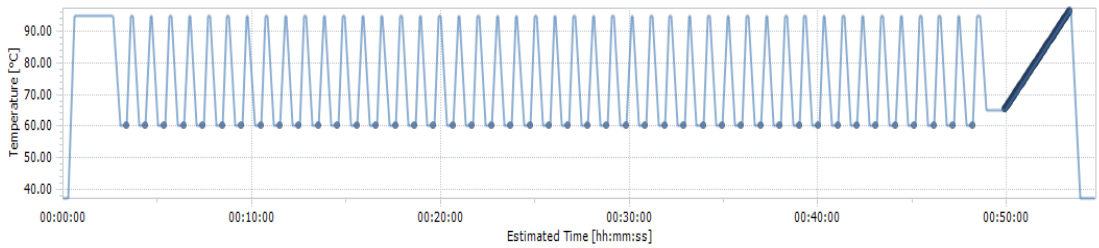

Figure 19 - Graphic representation of qPCR protocol for the geNorm reference gene data collection.

Non-template controls (NTCs) were run on all plates and for all genes to control for non-specific amplification. An amplification signal in a NTC well would indicate a potential contamination issue or an issue with the primer. Following collection of data, processing and subsequent selection of references genes was performed using the GeNorm algorithm to assess the stability of each gene for use as a reference value(374–376). The fifth generation of the algorithm was used as incorporated into qBase+ software (Biogazelle, Gent, Belgium). The software excluded blank qPCR plate wells and removed anomalies (defined as outliers within a sample triplicate). Results from the GeNorm analysis are displayed in Figure 20, Figure 21, and Table 15.

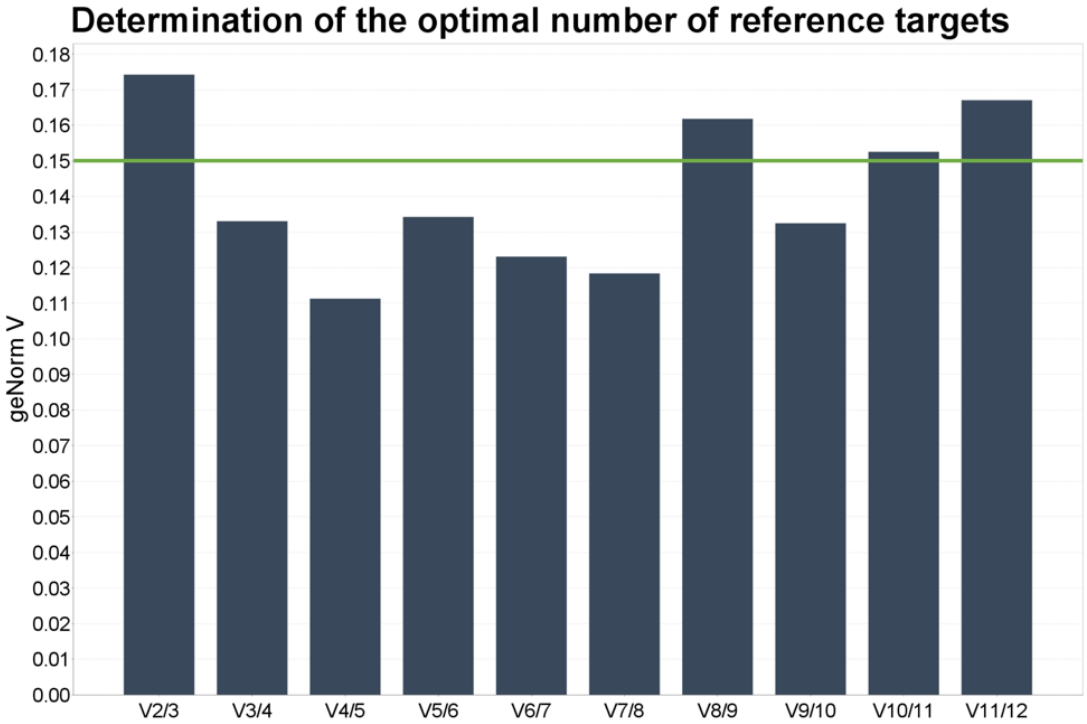

Figure 20 – GeNorm V analysis results: Determination of the optimal number of reference targets. The green line represents the algorithmic threshold, with a desired result below the line. From the results, the optimal number of reference targets is 'V3/4' defined as three reference targets.

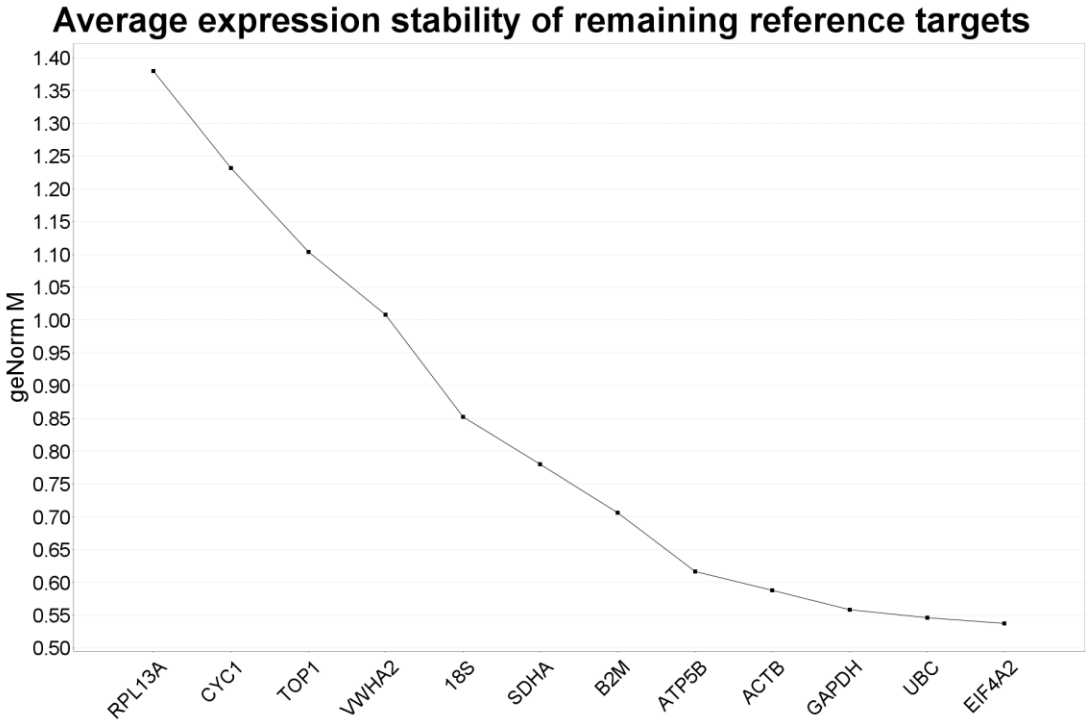

Figure 21 – GeNorm M analysis results: Average expression stability of remaining reference targets. A lower value indicates less variation across samples and greater stability. From the results displayed in Figure 20, the selection was then made of the 3 reference targets exhibiting the greatest stability, namely GAPDH, UBC, and EIF4A2.

Table 15 - GeNorm V interpretation results for qPCR reference gene evaluation for study samples. Output generated from qBase+ software.

geNorm V Interpretation	
Data	Interpretation
Input	geNorm analysis was initiated on 12 samples and 12 reference targets.
Missing values	The following reference target(s) contain missing data and were excluded for geNorm analysis: None.
Experiment design	Not all samples were measured in the same run for a given reference target. As inter-run variation might contribute to decreased expression stability, a proper geNorm analysis requires that all samples are measured in the same run for a given target (i.e. sample maximization strategy according to Hellemans et al., Genome Biology, 2007 (377)). The built-in inter-run calibration procedure in qbase+ does not correct for this.
Results: Optimal reference target selection	The **optimal number of reference targets** in this experimental situation is **3** (geNorm V < 0.15 when comparing a normalization factor based on the 3 or 4 most stable targets). As such, the optimal normalization factor can be calculated as the geometric mean of reference targets **GAPDH, UBC and EIF4A2.**
Reference target stability	Medium reference target stability (0.5 < average geNorm M ≤ 1.0). This is typically seen when evaluating candidate reference targets on a heterogeneous set of samples (e.g. treated cultured cells, cancer biopsies, or samples from different tissues). More reference values in Table 1 in Hellemans et al., Genome Biology, 2007 (377).

The algorithm, as summarised in Table 15, evaluates different aspects of the candidate reference gene data. The main two factors are the coefficient of variation (geNorm V or CV), allowing identification of the optimal number of reference genes by the reliability of the normalisation, and the medium reference gene cross-sample stability (geNorm M). Homogenous sample panels should show values of less than 0.25 for CV and 0.5 or below for M, whereas heterogenous panels should show respective values less than 0.50 and 1.0 (377). The sample panel showed a CV value of 0.133 and ~0.55 for M. Despite M being marginally outside the recommended threshold for homogenous samples, it was within tolerance compared to the heterogenous suggested values. The low CV value reassured this assessment as it was much lower than that for the recommended homogenous panel threshold.

From these experimental results, GAPDH, UBC, and EIF4A2 were selected as the reference target genes of interests.

Laboratory Methodology

Biopsy processing

After transfer from theatre, biopsies were rapidly processed. The laboratory apparatus for processing was set-up prior to attending the surgical theatre for sample collection. Two pathways were established for sample preservation. These were necessary to enable sample protection before subsequent laboratory analysis. Separate processing routes were established to provide back-up options and to allow differing analyses to be performed. However, the robust back-up preservation method limited certain analyses being used. The fixed tissue could not be analysed for enzyme activity, but the primary snap-frozen tissue would allow this.

The samples were initially divided into multiple smaller pieces using a scalpel. This allowed for multiple sub-samples from the outset and was necessary for efficient sample preservation. At this stage, they were also trimmed of any remaining connective tissue or fatty tissue as this was not the target tissue. The muscle pieces were separated before preservation processing, with the majority going towards the snap freezing route and the remainder into formalin fixation and paraffin wax embedding destination. The snap freezing laboratory set up is demonstrated in Figure 22. The formalin fixation route similarly began with steps A and B but continued with 24-hour immersion in 4% buffered formaldehyde. This is pictured in the bijou on the left of Figure 22, image A and is described in further detail later.

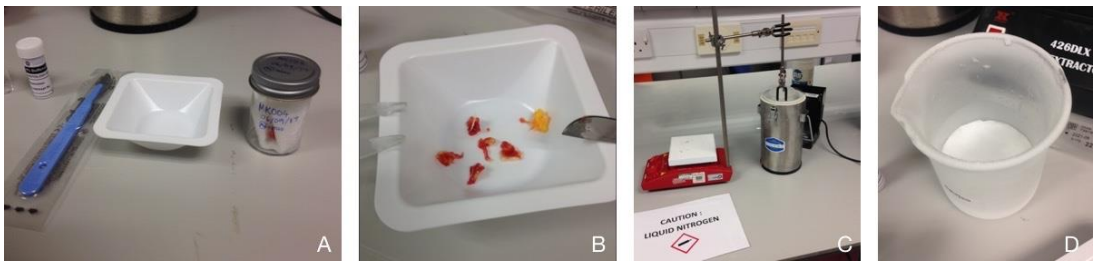

Figure 22 - Snap freezing of muscle biopsies in the laboratory. A) Bench set-up with sterile equipment and container with biopsy fresh from theatre. B) Cutting of biopsy into smaller pieces to aid sample preservation, remove other tissue types, and allow multiple analyses per sample. Size 22 Swann Morton scalpel blade for scale. C) Cooling of isopentane beaker in dewar of liquid nitrogen. D) Beaker of frozen isopentane on bench. Biopsy samples were immersed immediately as the isopentane thawed and allowed to snap-freeze for 20-30 seconds. Extraction fan present to prevent respiratory tract irritation from vapor exposure

Snap freezing of the muscle samples was performed using isopentane (2-methylbutane) that was cooled in liquid nitrogen (LN_2). Isopentane was cooled until

it changed to solid state indicated by a distinct visual change to opacity. It would then be removed from the LN_2 and be allowed to warm up on the lab bench until it thawed. At this temperature of around -160°C muscle samples pieces would be dropped in to the liquid for 20-30 seconds while they snap froze (322,326,327,343,378). The freezing process changed the muscle colour from red to pink. The snap freezing method in isopentane preserved the samples in a fresh state whilst also preserving sample architecture (378).

The snap frozen samples were then transferred to subject-specific bijous containers cooled on dry ice and subsequently stored in a -80°C ultra-low temperature freezer. Samples preserved and stored in this way were to be used for the majority of laboratory analyses.

The remainder of the samples were submerged in 4% buffered formaldehyde for 24 hours for fixation. They were then transferred to 70% ethanol (30% dH_2O) prior to embedding in histological paraffin wax blocks. This final step of wax embedment was performed by the local Shared University Research Facility (SuRF) histological service. A request was made for transverse muscle fibre orientation in the wax blocks to aid with later imaging and analysis. Though limiting certain analytical techniques from being used on samples stored in this way, this storage method allowed for a back-up biopsy component to be retained. Examples of excluded potential analyses included metabolic tests, cytometry, some genetic work, and some immunological targets due to confounding protein cross-linking. These samples could then be stored reliably at room temperature within the laboratory environment until further analysis.

Histochemical analysis

Histological analysis to determine anatomical structure and protein expression was performed with a variety of assays. Initial histological staining targets allowed for determination of sample anatomical and structural orientation, supplemented with immunological myogenic determinant and structural markers.

Cryo-sectioning and slide mounting

Frozen samples were cut into section using a Bright Cryostat (Standing Refrigerated Microtome – OTF/AS/MRID/MGII). The cryostat was set to -20°C, 20 degree cut angle, 8μm thick sections, and a fresh blade was used from the start of the project. As the area of the blade became used, the blade was moved sideways to keep the cutting-edge location sharp.

Samples were transferred from -80° freezer storage to the cryostat on insulated dry ice. A chosen sample would be placed on top of a cryostat chuck block and encased in OCT.

Sections were cut using the manual function of the machine, and serial sections were cut where possible. Occasional readjustment of the machine or a poorly cut or rolled section prevented this in all cases. At times a deep cut was made into the tissue to provide a chance to cut sections from a different part of the biopsy and provide a wider representation of the sample. Due to the variable thickness of the samples, this was not always possible. The use of the built-in 'Easi-set anti-roll plate' component aided successful sectioning by controlling for static.

All slides were mounted with four sections on Superfrost Plus Gold Adhesion slides. With analyses planned in triplicate, this extra section allowed for a margin regarding mounting errors or staining protocol disruptions.

Histochemical Staining Analysis

Haematoxylin and Eosin stain was used to identify microscopic anatomy for purposes of orientation. This pre-empted staining with the further targeted immunohistological panels.

Immunofluorescence staining for fibre type

Muscle Heavy Chain 7 Immunofluorescence Panel

Immunofluorescence staining was utilised to determine patient skeletal muscle fibre type compositions from their biopsy tissue samples.

Biopsy slide sections were fixed for 6 minutes in methanol at -20°C. Sections were washed 3 times using phosphate buffered saline with Tween20 0.05% (PBST) for 3 minutes per wash. These steps permeabilised the sections and removed remaining fixative. Slide sections were ringed with hydrophobic PAP pens (Vector ImmEdge) to aid with liquid retention during the incubation steps. Sections were blocked using DAKO Protein Block (x090930) for 15 minutes to reduce background staining from endogenous proteins, before primary antibody (MyH7 (Santa Cruz – SC53090)) incubation at 1:500 concentration in DAKO Ab Diluent (s202230) at +4°C overnight.

Sections were washed in PBST three times for 3 minutes per wash, followed by another blocking step, before secondary antibody (donkey-anti-mouse TRITC (Fischer-Scientific – PA128625)) incubation at 1:750 concentration in DAKO Ab Diluent (s202230) for 1 hour at +37°C.

Following secondary antibody incubation, sections were washed 3 times with PBST, and once with phosphate buffered saline (PBS). Sections were mounted and counterstained with Vectashield Antifade Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, CA, USA – H-1200), then coverslips were applied and sealed with nail polish.
The full protocol and materials list can be found in the Histology appendix.

Microscopy System and Slide Imaging

Microscope imaging was performed using a Nikon E800 fluorescence microscope at 100X and 200X magnification. Microscopy was completed within 24 hours of an immunofluorescence staining. Slides were kept at +4°C when this could not be completed same-day. This was performed to prevent fluorescence fading.
Digital imaging was performed with a Nikon DS-L4 microscope camera control unit tablet and a Nikon DS-Fi3 camera. Images were taken across all channels and later digitally merged with separate software. While invisible to the naked eye, the camera allowed detection of Cy5 where used. The microscopy imaging apparatus was regularly serviced by a Nikon technician who calibrated the system for the desired panel. All images were transferred from the tablet to a separate computer for processing.

Imaging was performed in the FITC channel alongside the target DAPI and TRITC channels. Despite the lack of labelled fluorophore in this wavelength, the autofluorescence of structures assisted with subsequent muscle fibre border distinctions.

Digital Image Processing

Digital image processing was conducted within ImageJ software (NIH, USA). Muscle fibre characterisation was clearly identified by the immunofluorescence staining of the structures. Type 1 skeletal muscle fibres, as expressers of MyH7 were labelled brightly in the chosen TRITC fluorophore channel. Type 2 fibres remained darker as they do not express the fluorescently labelled protein. Fibre boundaries were marked with identifiable nuclei, sarcolemmata and basement membranes.

All measurements were performed in triplicate. This occurred for each patient sample and for each section. During slide mounting, four sections were initially located on each slide and labelled ‘A’-‘D’. Where a section remained and there was strong reason to believe staining was successful, sections ‘A’, ‘B’, and ‘C’ were analysed. ‘D’ was included when there were issues with one of the first three

sections, such as section detachment during staining. This ensured uniform triplicate section analysis across all samples.

Measurement of the lesser diameter of the muscle fibre prevented any distortion artefact from influencing analysis (Figure 23). This was identified by creating the largest possible circle within a fibre, and then measuring the circle diameter. For each section, three fibres of each type were measured to deduce a reliable average. Visually typical examples spanning the range of sizes from each section were selected to provide representative values. This provided nine measurements of fibre lesser diameter per patient.

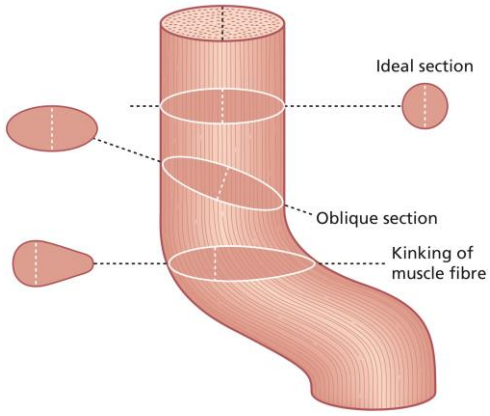

Figure 23 - Demonstration of lesser diameter measurements and the impact of oblique sections and fibre kinking. Figure from Muscle Biopsy - Dubowitz 2013 (322).

The number of each fibre type were totalled and recorded. This provided three measurements of each type of fibre per patient. Due to the fixed nature of the standardised size of the field of view, a larger fibre diameter resulted in a lower number of observations overall. To allow meaningful and straight forward comparison of the data, the number of fibres observed in each type was therefore reported as a ratio (type 1: type 2) and separately as percentages where relevant. The metric provided insight into the metabolic phenotype of the patients' muscle samples. Where necessary, the FITC channel image was used to contribute to the merged image to provide clear fibre border distinctions (Figure 24).

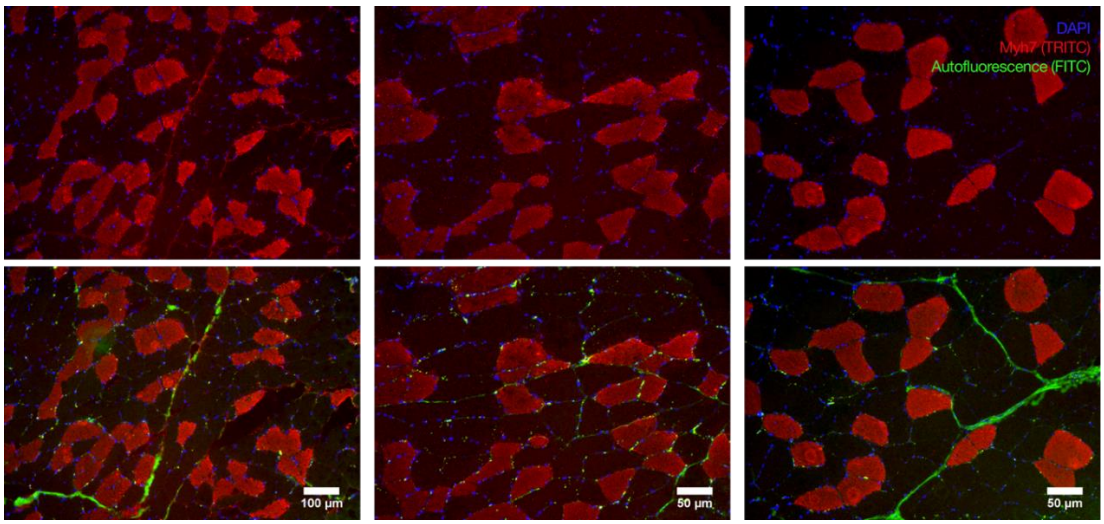

Figure 24 - Merged immunofluorescence Myh7 panel. Top images show only DAPI and TRITC channels. Bottom images show additional third channel merge (FITC autofluorescence from connective tissue) which was used to aid in distinguishing fibre boundaries.

Collected data were analysed further using the Microsoft Excel software (Microsoft Corp., WA, USA). Measured data from the ImageJ analyses were inputted to spreadsheets and processed to calculate averages and ratios to be later used in results analysis.

Quantitative Real-Time Polymerase Chain Reaction (qPCR) analysis

The quantity of mRNA for each target gene were analysed in each sample. This provided insight into the protein synthetisation and expression of each representative genetic marker. Higher expression of a representative gene signified activity that could be contextualised in terms of patient muscle physiology and selected systemic physiology.

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines provide a standardised list of experimental detail to be included in quantitative real-time PCR experiments publication data (379). The desired information seeks to improve transparency of experimental design, quality of laboratory results, and to improve study reproducibility. This study followed the MIQE guidelines to adhere to these principles.

RNA extraction

Each skeletal muscle sample was lysed followed by isolation of its RNA with the Trizol method (364–366). To improve stability of the product, RNA isolate was reverse-transcription synthesised to cDNA. With this product, the use of primers targeting the relevant genes of interest (GOIs) could be used to identify the relative quantities of expression of each gene using the qPCR technique. The RNA extraction, reverse-transcription, and qPCR full protocols are listed in the Molecular Protocols appendix.

NanoDrop analysis

Before running sample analyses on the NanoDrop ND-1000 instrument, a blank test was run using DEPC/RNase free water. This was repeated every 10 samples. Between every sample, the measurement surfaces were wiped with lint-free laboratory tissues (Kimwipes, Kimberley-Clark, TX, USA). 1μl of sample was loaded onto the pedestal for each measurement. The instrument was regularly tested to ensure appropriate hydrophobic properties were present to allow the samples to bead on the measurement platform and allow reliable analysis.

Trizol RNA Extraction Method

The Trizol RNA extraction method followed the included kit protocol (TRIzol reagent - Invitrogen, CA, USA; 15596026). All consumables were certified molecular grade (Human DNA, DNase, RNase, PCR inhibitor-free) and all surfaces were cleaned with Trigene (Tristel, UK) or RNAseZap surface wipes (Thermo Fisher Scientific, MA, USA). All possible consumables and instruments were autoclaved before use. Fresh filter pipette-tips were used, and personal protective equipment (PPE) gloves were frequently changed.

Biopsy samples were transferred on dry ice from freezer storage to the laboratory benchtop. Samples were transferred from storage bijous into a cooled sterile container. Samples were cut into uniform size of approximately $3mm^3$ and 40mg. All remaining sample was transferred back to the storage container. While frozen, the samples were minced using a scalpel, with care taken to contain the process. Samples were transferred to 2ml Eppendorf tubes on wet ice.

1ml of Trizol reagent was added to the Eppendorf tube where the mixture was homogenized using a pellet pestle cordless motor (Sigma Aldrich; Z359971) for 2 minutes on wet ice. Samples were then incubated and triturated 20X for 5 minutes at room temperature. At this stage, 200µl of chloroform was added to the Eppendorf tubes, which were vigorously shaken for 20 seconds. They were then incubated at room temperature for 3 minutes, followed by centrifugation at 12000g for 15 minutes at 4°C.

The samples separated into their three phases; aqueous phase (containing RNA), inter-phase (containing DNA), and organic phase (containing protein). The aqueous phase, roughly 600µl, was carefully removed by pipette to a 1.5ml Eppendorf tube, to which 500µl of isopropanol (Fisher Scientific; BP2618) was added. The sample was incubated for 10 minutes at room temperature. The inter-phase was also removed and stored at -80°C for later potential analysis.

The sample RNA mixture was then centrifuged at 12000g for 10 minutes at 4°C to concentrate a pellet of RNA at the tube base. The suspension was removed by a quick inversion over a waste container, while the pellet remained in the tube. 1ml of cold 75% ethanol (25% RNase free-dH_2O) was added, prior to vortexing and centrifugation at 7500g for 5 minutes at 4°C to wash the pellet of contaminants and re-concentrate the pellet. The suspension was removed via inversion after which the pellet was air-dryed for 15 minutes to allow remaining ethanol to evaporate.

Finally, 30µl of DEPC/RNase free cold water was added to the tube which was triturated and vortexed to dissolve the pellet. RNA analysis was performed with a NanoDrop to record absorbance ratios and yield. Storage until the next methodological stage occurred at -80°C. The full protocol and materials list can be found in the Molecular Protocols appendix.

Genomic DNA Elimination

The extracted sample RNA was treated to remove of remaining genomic DNA (gDNA) from the sample. Without the removal of gDNA, the qPCR results may have provided false positives by showing higher amplification during earlier cycles. While not an explicit wash for removing phenols or alcohols, the buffering process provided some control for pH values. Later steps addressed the issue of potential contamination of that kind.

To remove potential gDNA contamination, a Precision DNase kit (Primer Design, UK; DNASE) was used. Sterile equipment was used for all steps. Initially, as samples were thawed on wet ice, the kit buffer and enzyme components were combined. Then 4µl of the kit solution was added to each sample, triturated briefly, and vortexed. Samples were then incubated for 15 minutes at 30°C on a heat block to activate the DNase enzymes and degrade any remaining gDNA, followed by incubation at 55°C for 5 minutes to inactivate the DNAse. Samples were transferred to wet ice and subsequently were stored at -80°C. The full protocol and materials list can be found in the Molecular Protocols appendix.

Measurement of NanoDrop ratios and yield was performed for all samples. The RNA extraction yield ranged from 113ng/µl to 797ng/µl, however the purity of the samples was found to range from 1.30 to 1.97 for the 260/280 ratio. A sample without genomic DNA contamination would record a NanoDrop 260/280 ratio of 2.

RNA Quality Analysis

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines require the adherence to one quality control (QC) step(379) during initial extractions, which was covered by the reproducible NanoDrop analyses. Further quality steps were subsequently performed throughout the qPCR experimental process.

RNA to cDNA

Synthetic complementary DNA (cDNA) was created from the extracted sample RNA. This was made to stabilise the extracted nucleic acid product due to the fundamental quality threat to the extracted RNA from the abundance of RNases. RNases are difficult to eliminate from the laboratory environment despite extensive routine cleaning Subsequent experimental analysis steps would expose the nucleic acid to harsher conditions which could damage their integrity and impact results.

Synthesising of cDNA was performed with a High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, MA, USA; 4368814). All study samples were diluted to 100ng/µl concentration using molecular grade water prior to performing the synthesis. The included product kit protocol was followed. Kit reagents and components were combined, 10µl of kit master mix was added to each sample, and the samples were run on a thermal cycler in 4 steps. Initially samples were brought to 25°C for 10 minutes, then 37°C for 120 minutes, then 85°C for 5 minutes, and finally down to 4°C to finish the program. The thermal cycles were completed on a Veriti Dx 96-well machine (Thermo Fisher; 4452300). NanoDrop analysis for ssDNA was performed following the reverse transcription protocol. 260/280 values were all >1.77, 260/230 values >2.16, and yields >1580ng/µl. Due to the different nucleic acid composition of the cDNA compared to RNA, the ratio benchmark values are slightly higher. The values observed represented consistency

and quality across all samples. At this stage, further dilutions were made to ensure consistency alongside conformity with the required starting concentration for the qPCR technique of 5ng/μl of cDNA. This was performed in two dilutions by a factor of 10, followed by assessment of values with the NanoDrop instrument, and a final dilution of 1 in 4. At every stage appropriately calculated dilutions were made for uniform concentration across the samples. Samples were then stored at -20°C. The full protocol and materials list can be found in the Molecular Protocols appendix.

Real-Time Polymerase Chain Reaction: Genes of Interest

With stable cDNA generated, the qPCR technique could now be used to analyse the study samples(380) by comparing expression of genes of interest again the established reference gene target of EIF4A2, UBC, and GAPDH. Having identified the reference gene targets, these were now run against all 59 study samples. The amplification thermal cycle protocol remained the same as that used for the reference genes, displayed in Methodology Development Reference Gene Selection (Table 14). Once again, non-template controls (NTCs) were run on all plates. Samples were run in triplicate. The LightCycler 96 instrument detailed program methodology output and settings are displayed in the Molecular appendix section.

Following the collection of reference gene data, the protocol was run once more for each of the experimental GOIs. Plates were run for the myogenesis marker panel of Pax7, MyoD1, Myf5, and Myog, and for the senescence (CDK2NA) and inflammatory marker panel of IL6, and TNF.

During the qPCR experimental runs, the instrument detected the fluorescence of the amplified PCR product, providing a representation of the quantity present. With each PCR cycle, the quantity of primer-bound target DNA sequence doubled. The lower the number of cycles it required to amplify the product to a quantitative cycle threshold value (C_q), the larger quantity of the GOI was present in the initial sample. This value allowed for comparison of sample GOI expression levels once raw data were processed.

qPCR Data Processing

The data output from the Roche LightCycler 96 required several processing steps to contextualise the raw output and to ensure results quality. Initial proprietary file formats were analysed using LightCycler 96 Application Software (F Hoffman-La Roche AG, Basel, Switzerland) giving access to both raw data and pre-generated results graphics. These integrated analysis steps aided in identifying any confounding results generated during the process. Dissociation melting curve analysis was a key quality step to determining correct product amplification. Finally, exported data were further processed using Excel software (Microscoft, WA, USA).

The quality of the qPCR results was carefully assessed. Melting curve data were examined for non-specific amplification and to ensure that the desired product was present. This also ensured that the previous DNase genomic DNA elimination step was effective.

Following this, sample triplicate outliers were excluded, with a threshold of 0.5 cycles, to reduce PCR replicate variation. The non-template controls (NCTs) were examined for amplified product which would indicate contamination of the master mixes or primer-dimer amplification. Following these steps, the ΔC_q levels could be calculated using the following formula:

$$\Delta \mathrm{Cq} = \overline{Cq}_{GOI} - \overline{Cq}_{Reference\ Genes}$$

Relative expression values (ΔC_q) are calculated by determining the difference in cycle number between the target gene expression level and the average expression level from the housekeeping (reference) genes. Each housekeeping mean was unique to each sample. This was performed due to small variation in the starting quantities of sample RNA. Despite the standardisation of biopsy size that was lysed in the protocol, this subsequent step provided further control.

Following the qPCR program, a thermal ramping program was run to identify the melting temperature of the PCR product. The melting curve of the products showed

the temperature when the double stranded product separated into single stranded DNA. The exact temperature for each target Gene of Interest (GOI) PCR product varied depending on the contained quantity of GC complexes; with more resulting in a higher melting temperature(381). Anomalous qPCR results were identified and eliminated based upon outlying melting temperatures which are usually lower than the main GOI PCR product. Supplier primer validation was performed on a variety of tissues, however specificity was assured. For example, anomalous qPCR results may include intramolecular 'hairpin' amplification of primers to themselves which have a lower melting peak. An example of the identification and exclusion of these data points is demonstrated in (Figure 25) as performed for plate 2 of the UBC reference gene.

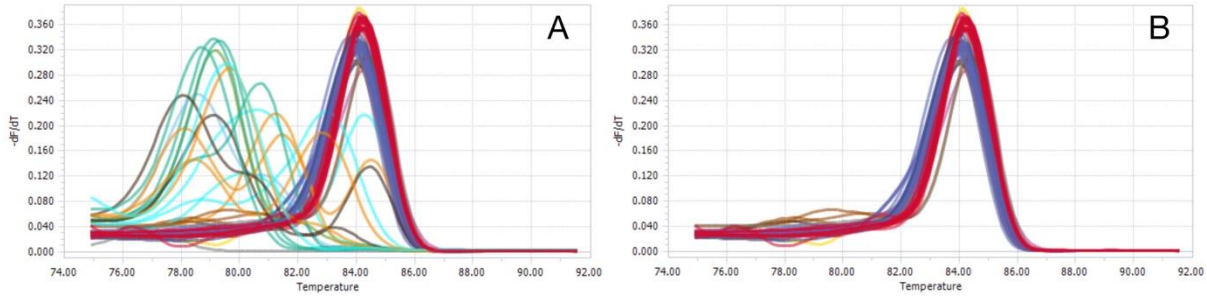

Figure 25 - Identification and elimination of the outlier qPCR products using melt curve analysis. Graphs represent data from the UBC gene qPCR experimental runs. A) Pre-processing with 83 positive wells. B) Post-processing with 66 positive wells remaining. The excluded 17 samples were instead correctly classified as having non-detected PCR product confirmed by Cq values.

This process was performed for all qPCR results and functioned as a backstop check to counteract the issues encountered with earlier RIN analysis. It also identified any issues with primers created during this specific technique.

While cycle threshold quantification (C_q) values provided quantitative data, this was not the final step. A series of processes were used to clean the results from each qPCR run. These eliminated anomalous values which could not be deemed reliable. The exclusion criteria were primer quality issues (as evaluated by melting peak analysis), and triplicate outlier exclusion (indicating possible manual pipetting variation error). The latter steps were performed using exported data within Excel software. As stated, these were performed with a 0.5 cycle threshold.

NTC well data were examined using the same protocol and were all found to have no detected expression, showing that all positive experimental sample data was showing true amplification of GOIs and that there was no contamination.

The quality control protocol steps were rigorous and resulted in multiple exclusions with each quality control step. Some samples were excluded on the basis of having no viable housekeeping reference data, and therefore no normalising data, while others were excluded on a per gene target basis due to melt data anomalies or negative results.

qPCR Data Analysis

Following data processing, they could now be analysed. Mean C_q data were calculated from remaining values for each sample GOI.

Next, the differences of expression levels between the experimental target GOIs and the reference GOIs were calculated. The ΔC_q values were calculated from a subtraction of the reference gene mean C_q value from that of the experimental GOI C_q value. A lower value indicated higher expression of the target gene. The ΔC_q value provided the relative quantitative level of expression of each gene which was then incorporated into wider cohort comparisons.

Data Presentation and Statistical Analyses

The study results of individual variable measurements are presented in several formats. Univariate evaluation of temporal trajectory is performed. The relationship within each metric group category is also examined. While all metrics were analysed for their total response rate, some were collected in the full cohort, and some were collected only in a sub-cohort. This is reflected in the stated sample number. A snapshot cohort overview is presented in Table 16, with a fuller breakdown displayed in the following chapter.

Table 16 - Summary data for study sub-cohort recruitment and participation numbers. Full data capture for the study is detailed in the Results sections.

Characteristic	Cohort			
	All	Enhanced	Routine	WEL
n	**63**	**30**	**13**	**20**
	m (SD)	**m (SD)**	**m (SD)**	**m (SD)**
% Female	51	50	31	65
Age pre-op	65 (10)	64 (9)	65 (12)	67 (12)
BMI pre-op	32.4 (5.9)	32.7 (5.3)	31.9 (6.7)	32.4 (6.0)
Biopsy samples taken	58	27	11	20

Individual study variables measured over time are visualised using boxplot and Loess (local regression) smooth fit curves in figures to show trends. The variables were also statistically interrogated with omnibus One-way ANOVAs and, if found to show significant differences (alpha 0.05), were further examined with inter-time-point post-hoc pairwise comparison using t-tests with pooled standard deviations and Bonferroni-Holm (Holm) corrections for multiple comparisons (382,383).

These statistical tests and graphical figures were chosen based on the repeated measurements of the variables in the same sample population. Boxplots visualise the data distribution and the Loess regression accounts for differing sample size and the distribution of testing intervals. Pair-wise t-tests with corrections were chosen over Tukey pairwise comparisons due to sample size differences, and family-wise error rates were chosen for the same purpose. As the number of time-point comparisons was 5, the step-wise Holm corrections method was chosen over the Bonferroni alone corrections method to avoid overly conservative correction of p-values potentially resulting in type II errors. The tests assume homogeneity of variance, which was

confirmed with Levene's tests. Data normality was assessed with Anderson-Darling tests.

Physiological data were analysed to establish relationships using linear regression equations and Pearson and Spearman Rho correlation coefficients where appropriate. Correlation strengths are stated and are also defined by the r brackets: 0-0.2 "very weak", 0.2-0.4 "weak", 0.4-0.6 "moderate", 0.6-0.8 "strong", 0.8-1 "very strong" (384,385). Alpha was defined as 0.05.

Univariate and multivariate regressions modelling was used to examine the complex relationships between preoperative and perioperative factors and characteristics on study outcome measures. Variables were initially compared using Linear Regression Analysis and Best Subset Regression models however missing values created overfit bias with the latter technique. Instead, General-to-Specific multivariate regression modelling techniques were used (386). Sequential steps initially remove collinear variables before filtering out non-significant variables to arrive at a best-prediction model. Collinearity was assessed with variation inflation factors (VIFs) indicating how collinear factors served to artificially inflate R^2 values resulting in biased, overfit models. Non-significant variables were removed in stages with progressively conservative alpha values. The progressive method was used as missing values in different variables can result in models decreasing sample number as the general-to-specific modelling technique progresses. Overcorrection too early in the process could have led to an incorrect model erroneously omitting a key independent variable(s). The whole model alphas were set as 0.05.

Data were processed using Microsoft Excel 2016 (v16.0) and R Studio (v1.1.463). Graphics were created using the R package ggplot2 (v3.1.0). Statistical analyses were performed within Minitab 17 (v17.1.0) and R Studio.

Chapter 5: Results - The MAKRO Study

Overview: The Muscle Assessed Knee Replacement Outcome (MAKRO) Study

Study Flow and Demographics

Study recruitment occurred during a 17-week time window between August and December 2017. 116 patients listed for primary TKA were screened by members of the clinical team for study eligibility. Exclusions and logistical constraints left a remainder of 92 patients to be approached, of which 72 consented to study participation.

Participants cohort breakdown comprised of 35 for the Enhanced cohort, 17 for Routine, and 20 for the WEL cohort. Initial cohort numbers were marginally reduced due to several reasons such as cancellations due to hospital bed capacity. 4 patients were delayed by more than 6 months to outside of the study time-window and were therefore unable to be followed up.

Of those initially consented, 63 patients proceeded with total knee arthroplasty (30 Enhanced, 13 Routine, and 20 WEL participants). The surgical operations took place between September 2017 and February 2018. Follow-up research appointments occurred between October 2017 and March 2019.

The screening and recruitment processes are summarised as a CONSORT-style (387) clinical study flow chart in Figure 26.

Across the duration of the study, 2 participants withdrew, some were deemed lost to follow-up, and others were incorrectly withdrawn. Details for these are listed later in this section. Attempts were made to contact those lost in all cases.

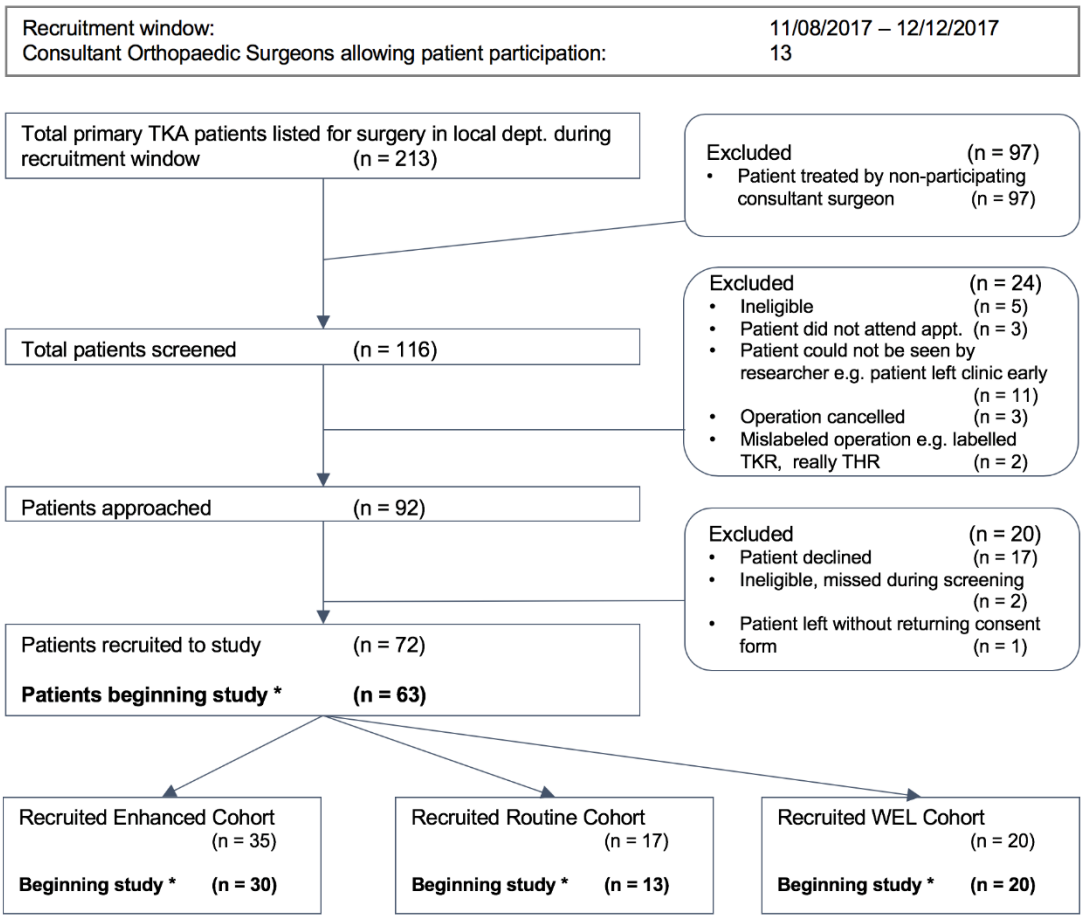

*Figure 26 - MAKRO Study CONSORT-Style Screening and Recruitment Flow Chart. Initial ineligibility criteria were stated in the study protocol, discussed in the methodology of this thesis, and excluded by clinical research staff as part of initial screening. * = Exclusion of withdrawal criteria for each cohort are listed and described separately per cohort later in this section.*

Whole Study Demographics

The patients recruited to the study broadly reflected the typical total knee arthroplasty population observed within the department and across the United Kingdom as represented in the National Joint Registry (200,388). For example, with female percentage (NJR 56.8%; MAKRO 50.8%), age (NJR 70 (IQR 64-76); MAKRO 65 (IQR 58-70)), and BMI (NJR 30.9; MAKRO 32.4).

For the purposes of full study cohort composition, the 9 patients who were subsequently excluded from data collection due to withdrawal, cancellation or deferral of operations were not included in this analysis. Therefore, cohort demographics represent the 63 patients who took part in the study. The 9 patients who were recruited to the study but did not participate had only provided very basic demographic data which was retained for the purpose of identifying bias. These patients had mostly elected to participate in the Enhanced cohort (5 participants),

were 44% female, were aged between 54 and 88 at time of recruitment, and had a mean age of 70. They had a mean BMI of 31.6 and a mean Kellgren & Lawrence osteoarthritic severity score of 1.8. This was broadly representative of the remaining cohort showing no inadvertent exclusion bias. No data from these comparative categories was available on the screened and excluded individuals due to ethical data access restrictions.

The summary data for the full cohort and sub cohorts are displayed in Table 17.

Table 17 - Summary data for full MAKRO cohort compared to sub-cohorts. Category means and standard deviations are displayed where relevant.

Characteristic	Cohort			
	All	Enhanced	Routine	WEL
n	**63**	**30**	**13**	**20**
	m (SD)	**m (SD)**	**m (SD)**	**m (SD)**
% Female	51	50	31	65
Age pre-op	65 (10)	64 (9)	65 (12)	67 (12)
BMI pre-op	32.4 (5.9)	32.7 (5.3)	31.9 (6.7)	32.4 (6.0)
Kellgren and Lawrence Osteoarthritis Score for Op-knee	1.8 (0.8)	1.9 (0.8)	1.8 (1.0)	1.8 (0.5)
Scottish Index of Multiple Deprivation Quintile	3.0 (1.3)	3.4 (1.3)	2.9 (1.4)	2.5 (1.0)

Comparison of headline demographic categories showed comparability. The routine cohort comprised of less female participants than the other sub-cohorts, while the WEL cohort had more. The Enhanced cohort included participants with least deprivation and the WEL contained the most, as measured by the Scottish Index of Multiple Deprivation (SIMD) (389).

The major baseline demographic categories represented in the full study cohort are displayed in Table 18.

Table 18 – Full cohort demographic factor frequency table for the MAKRO study.

Characteristic	n (%)
Patients	***63 (100)***
Biometrics	
Sex	
Male	31 (49)
Female	32 (51)
Age pre-op	
40-50	4 (6)
50-60	15 (24)
60-70	24 (38)
70-80	15 (24)
80-90	5 (8)
BMI pre-op	
20-24.9	6 (10)
25-29.9	15 (24)
30-34.9	21 (33)
35-39.9	14 (22)
40-44.9	6 (9.5)
45-49.9	1 (1.5)
BEI pre-op	
10-14.9	1 (2)
15-19.9	1 (2)
20-24.9	2 (3.5)
25-29.9	7 (12.5)
30-34.9	7 (12.5)
35-39.9	14 (25)
40-44.9	10 (18)
45-49.9	8 (14)
50+	6 (11)
Kellgren and Lawrence Osteoarthritis Score for Op-knee	
1	22 (35)
2	33 (52)
3	5 (8)
4	3 (5)
Lifestyle	
Scottish Index of Multiple Deprivation Quintile (2016)	
1	8 (13)
2	19 (30)
3	12 (19)
4	14 (22)
5	10 (16)
Occupation category	
Sedentary desk work	20 (32)
Manual labour work	41 (65)
Alcohol intake per week (units)	
Nil	16 (26)
Consumption within NHS guidelines (1-14)	31 (51)
Consumption above NHS guidelines (15+)	14 (23)
Tobacco Smoking History	
Current	3 (5)
Previous	18 (29)
Never	41 (65)
Peak previous regular physical activity (Tegner)	
Low (level 0-3)	15 (25)
Medium (level 4-6)	16 (26)
High (level 7-10)	30 (49)
Comorbidities	
Diabetes	9 (10)
Hypertension	22 (35)
Long term pharamaceutical use pre-op	
Paracetamol	46 (73)
NSAIDs	16 (25)
Opiates (including cocodamol and other minor and major opiates)	27 (43)
Previous Lower Limb Arthroplasty	
Contralateral Knee	35 (56)
Contralateral Hip	8 (13)
Ipsilateral Hip	7 (11)
Contralateral Ankle	3 (5)
Ipsilateral Ankle	4 (6)

The cohort displayed a normal distribution across the majority of biometric factors. Exceptions were observed in two instances. Firstly, in the bioelectrical impedance values, where the instrument classified all values over 50 as the same, and secondly with the Kellgren & Lawrence (K&L) osteoarthritic clinical score, where most patients (87%) were classed as having mild symptoms. The low K&L scores were attributed to a lack of osteophytes amongst the patient pre-operative radiographs and their prominence in the K&L score over joint space narrowing (390). Knee joint space narrowing has shown to have greater correlation than osteophyte prominence with pain scores (391).

The comorbidities of diabetes and hypertension are reported as they showed the highest prevalence in the cohort. Comorbidities only present in very small numbers of patients were not reported due to their incidence being too small to attribute meaningful analysis, for example in 2 or less participants. No participants were diagnosed with any excluding comorbidities.

Across lifestyle factors, several notable observations were made. The majority of participants had an occupational history in manual labour (65%). The majority of participants either consumed alcohol within NHS recommended volumes or completely abstained (77%), and the vast majority of participants were not current smokers (94%) however some had previously been smokers and had stopped (29%). Lastly, the previous regular activity and sporting levels of the participants was skewed towards the higher end, with 49% of participants having previously taken part in regular high-level activity classified by a Tegner Activity Scale level of 7 and above. This level is defined as those who had regularly participated in competitive individual sports such as running or tennis, or in recreational field sports such as football, rugby, or hockey. Higher levels encompassed competitive impact or contact sports, and individual or team sports at national and international elite level.

The other demographic cohort categories correlated with routine expectations of the clinical demographic based on National Joint Registry data. The patient history of a 56% rate of previous contralateral knee arthroplasty was notable as high in the cohort. This may have affected the recent level of activity of those patients in the

cohort, and also provided them with a comparison of expectations based on their previous experience. It may also have had a positive influence on the study recruitment rate due to the uncertainty surrounding a first arthroplasty not being present.

Study Sub-cohort Demographics

Study participants elected to provide different commitment levels and volumes of data categorised by the study sub-cohorts. These were self-selecting and allowed for the maximum possible volume of data collection while respecting the different individual levels of perceived research burden alongside the routine clinical service burden. It was important to establish that the different cohorts were not distinctly different when the demographic factors were examined.

The data collection categories spanned all three sub-cohorts to varying extents. PROMs data were collected from all participants and analysed together, functional data were available for some time-points from both the Enhanced and Routine cohorts, and the extra time-point and additional functional data were only collected from the Enhanced cohort participants.

A comparison of characteristics between the cohorts was necessary to identify any overt skewing of the cohort demographics, while keeping an awareness that the entire cohort was itself a sample of the population. This is addressed in the following sections.

Enhanced Sub-cohort Demographics

The enhanced sub-cohort attended more clinics and provided more data compared to the rest of the cohort. While by nature a smaller sub-cohort, a comparison of demographic factors was made to those from the overall cohort to determine representation. These are displayed, along with comparison to the other sub-cohorts, in Table 19.

Table 19 - Sub-cohort demographic factors frequency table with the MAKRO study. P values for similarity between sub-cohorts measured by One-Way ANOVA are shown for each sub-cohort compared to the entire cohort and also for the entire group.

Characteristic	Cohort			
	All	**Enhanced**	**Routine**	**WEL**
n	**63**	**30**	**13**	**20**
p value (One-Way ANOVA)	**Group: p=0.98**	Indiv. to All (p=0.99)	Indiv. to All (p=0.92)	Indiv. to All (p=0.74)
Biometrics	n (%)	n (%)	n (%)	n (%)
Sex				
Male	31 (49)	15 (50)	9 (69)	7 (35)
Female	32 (51)	15 (50)	4 (31)	13 (65)
Age pre-op				
40-50	4 (6)	0	2 (15)	2 (10)
50-60	15 (24)	9 (20)	3 (23)	3 (15)
60-70	24 (38)	15 (50)	2 (15)	7 (35)
70-80	15 (24)	4 (13)	5 (38)	6 (30)
80-90	5 (8)	2 (7)	1 (8)	2 (10)
BMI pre-op				
20-24.9	6 (10)	2 (7)	3 (23)	1 (5)
25-29.9	15 (24)	5 (17)	3 (23)	7 (35)
30-34.9	21 (33)	13 (43)	2 (15)	6 (30)
35-39.9	14 (22)	8 (27)	3 (23)	3 (15)
40-44.9	6 (9.5)	1 (3)	2 (15)	3 (15)
45-49.9	1 (1.5)	1 (3)	0	0
BEI pre-op				
10-14.9	1 (2)	0	1 (8)	0
15-19.9	1 (2)	0	1 (8)	0
20-24.9	2 (3.5)	0	1 (8)	1 (5)
25-29.9	7 (12.5)	3 (10)	1 (8)	3 (15)
30-34.9	7 (12.5)	5 (17)	1 (8)	1 (5)
35-39.9	14 (25)	8 (28)	4 (31)	2 (10)
40-44.9	10 (18)	6 (21)	2 (15)	2 (10)
45-49.9	8 (14)	4 (14)	1 (8)	3 (15)
50+	6 (11)	3 (10)	1 (8)	2 (10)
Kellgren and Lawrence Osteoarthritis Score for Op-knee				
1	22 (35)	10 (33)	7 (54)	5 (25)
2	33 (52)	16 (53)	3 (23)	14 (70)
3	5 (8)	2 (7)	2 (15)	1 (5)
4	3 (5)	2 (7)	1 (8)	0
Lifestyle				
Scottish Index of Multiple Deprivation Quintile				
1	8 (13)	2 (7)	2 (15)	4 (20)
2	19 (30)	7 (23)	5 (38)	7 (35)
3	12 (19)	6 (20)	1 (8)	5 (25)
4	14 (22)	8 (27)	2 (15)	4 (20)
5	10 (16)	7 (23)	3 (23)	0
Occupation category				
Sedentary desk work	20 (32)	8 (27)	3 (23)	9 (45)
Manual labour work	41 (65)	22 (73)	10 (77)	9 (45)
Alcohol intake per week (units)				
Nil	16 (26)	4 (13)	4 (31)	8 (40)
Consumption within NHS guidelines (1-14)	31 (51)	19 (63)	6 (46)	6 (30)
Consumption above NHS guidelines (15+)	14 (23)	7 (23)	2 (15)	5 (25)
Tobacco Smoking History				
Current	3 (5)	2 (7)	0	12 (60)
Previous	18 (29)	7 (23)	4 (31)	6 (30)
Never	41 (65)	21 (70)	8 (62)	2 (10)
Peak previous regular physical activity (Tegner)				
Low (level 0-3)	15 (25)	6 (20)	4 (31)	5 (25)
Medium (level 4-6)	16 (26)	7 (23)	3 (23)	6 (30)
High (level 7-10)	30 (49)	17 (57)	5 (38)	8 (40)
Comorbidities				
Diabetes	9 (10)	3 (10)	2 (15)	4 (20)
Hypertension	22 (35)	11 (37)	3 (23)	8 (40)
Long term pharamaceutical use pre-op				
Paracetamol	46 (73)	20 (67)	9 (69)	17 (85)
NSAIDs	16 (25)	8 (27)	4 (31)	4 (20)
Opiates (including minor and major)	27 (43)	14 (47)	4 (31)	9 (45)
Previous Lower Limb Arthroplasty				
Contralateral Knee	35 (56)	19 (63)	7 (54)	9 (45)
Contralateral Hip	8 (13)	3 (10)	5 (38)	0
Ipsilateral Hip	7 (11)	2 (7)	4 (31)	1 (5)
Contralateral Ankle	3 (5)	2 (7)	1 (8)	0
Ipsilateral Ankle	4 (6)	2 (7)	2 (15)	0

As can be observed in the comparison of the two demographic cohort factors tables, the demographic distribution is well preserved in the Enhanced sub-cohort. Similar observations were made between all cohorts, with demographic percentage representations tested with one-way analysis of variance statistical tests (p=0.98).

Surgical Operations and Recovery Period Study Flow

Participants' samples and data were collected in adherence to the protocol. Each sub-cohort encompassed different data collection structures and are therefore separated in their study flow reporting for this section.

Enhanced Sub-cohort

The Enhanced sub-cohort's successful data collection is displayed by category in Figure 27. Subsequent time-points and associated data categories are on the left of the figure, and the breakdown of any missed data is detailed on the right of the figure.

The initial recruited cohort of 35 was reduced to 30 due to a withdrawal and surgery deferrals outside of the study time-window. 3 patient samples were not taken, one due to the surgeon deciding not to do so based upon the stature of the patient, and 2 attributed to last minute changes in the operating list which were not conveyed to the researcher.

Following the first missed sample, changes were made in the biopsy collection protocol to create a greater presence within the clinical environment, such as checking informal surgical lists with clinical secretaries, and providing contact details to multiple theatre staff members in the case of a change of surgical time or date. While further samples were subsequently missed in other sub-cohorts, the change of protocol greatly reduced the number of these cases.

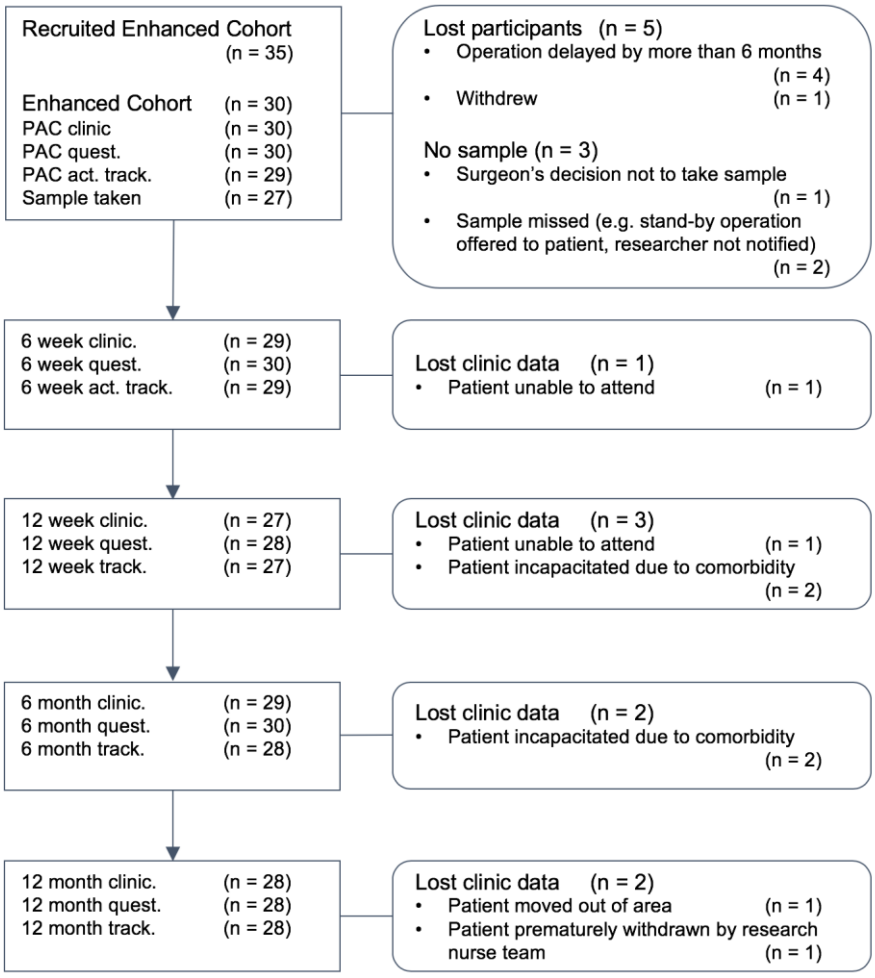

Figure 27 - MAKRO Study Enhanced Sub-cohort CONSORT-Style Screening and Recruitment Flow Chart.

A small number of patients were unable to attend certain time-points. Unattended appointments were rescheduled as soon as possible or, if not possible, postal questionnaires would be sent. At the 12-month time-point, one participant had moved out of area, and one was prematurely withdrawn rather than offered an alternative appointment when they called to say they could not attend.

Routine Sub-cohort

The Routine sub-cohort had a similar minor reduction in participant numbers but due to different reasons. 3 patients had their TKA surgical operation cancelled altogether, 1 patient planned to move out of area and so was transferred to the WEL sub-cohort, and 2 biopsy samples were not collected (Figure 28).

The questionnaire-only time-points at 12-weeks and 6-months post-op provided a reduced return rate than those filled out in the clinic. Routine cohort participants returned 65% of questionnaires at this time-point, whereas Enhanced cohort participants completed 97% of their questionnaires. Participants who failed to return questionnaires were contacted by phone to ensure that they had received them and were asked if they had any concerns with the forms. Some were uncontactable and others stated that the questionnaire had not arrived. Repeat questionnaires were sent in these latter cases, however this did not always guarantee a returned form set.

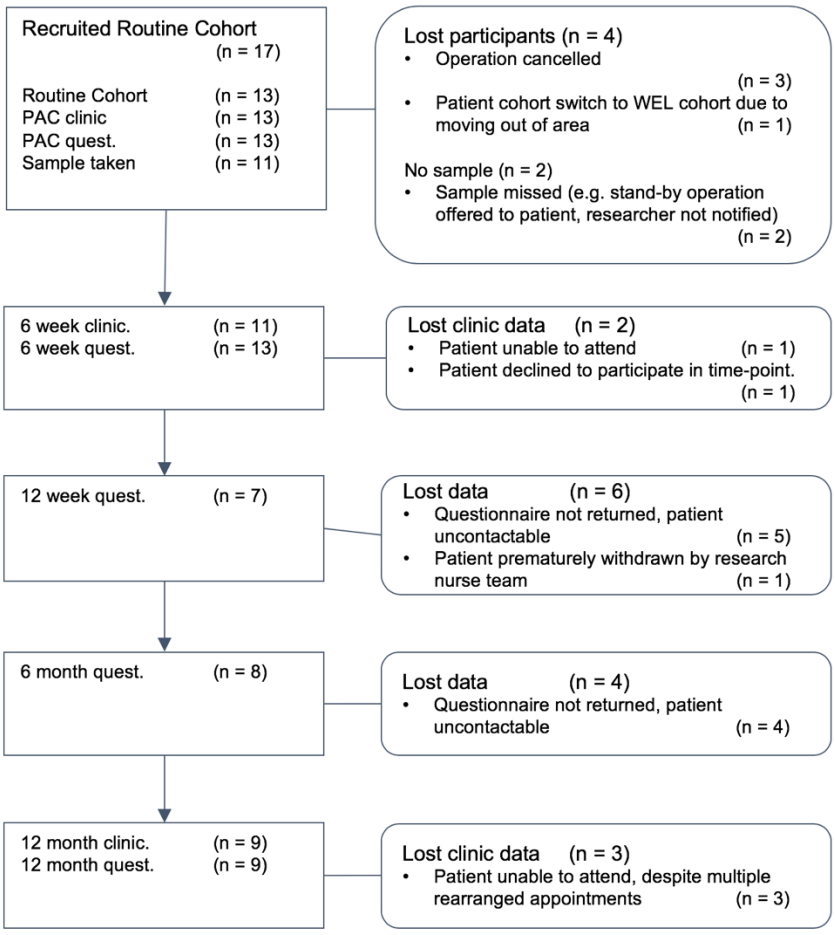

Figure 28- MAKRO Study Routine Sub-cohort CONSORT-Style Screening and Recruitment Flow Chart.

Two patients were unable to attend their 6-week follow-up appointment. Both filled in postal questionnaires as a substitute but did not record direct functional data.

At the end of the study, three patients repeatedly did not attend their hospital appointments. Due to the time-limited nature of the study, and the burden on the clinic frameworks, the patients were classed as lost data after 3 missed appointments.

West and East Lothian (WEL) Sub-cohort

The West and East Lothian (WEL) sub-cohort experienced a single participant reduction due to a withdrawal, and gained a participant due to a transfer from the Routine cohort (Figure 29). Biopsy samples were not taken in 2 participants, and follow-up questionnaire data return rate was similar to that observed in the Routine cohort. Four patients did not return any post-operative questionnaires and were uncontactable.

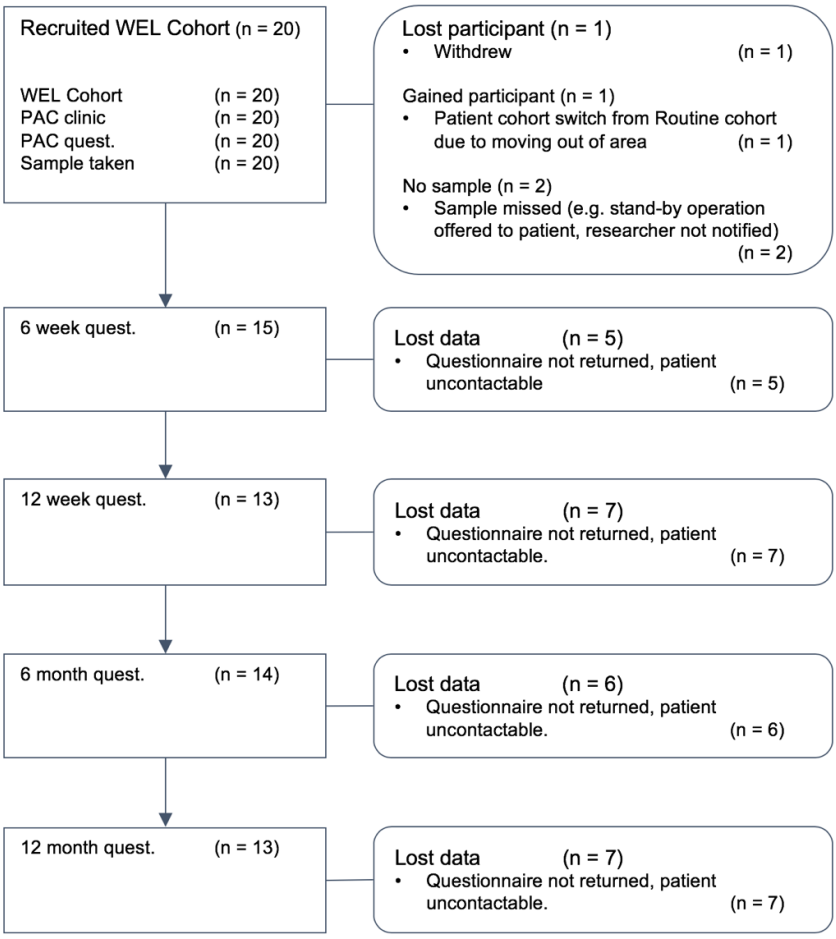

Figure 29- MAKRO Study West and East Lothian (WEL) Sub-cohort CONSORT-Style Screening and Recruitment Flow Chart.

Demographic and Clinical Baseline Factors

Out of the tabled demographic, lifestyle, and clinical baseline factors, almost all were used for subsequent investigations. Those criteria not used in analysis were excluded due to low numbers of participants. These comprised of the history of previous arthroplasty in joints other than the contralateral knee. This was decided due to the statistical implications and confounding factors arising from the inclusion of criteria with less than 3 participants per categorical variable. As such, the arthroplasty history for contralateral knee arthroplasty was the only arthroplasty history considered in further analyses. Though up to 8 participants had a history of other previous arthroplasties, due to the modelling methodology, which was dictated by the sample number of multiple factors, this reduced the viable sample numbers to below 3.

To allow for structured statistical analysis of demographic and clinical baseline factors, ordinal data categories were created from discrete and continuous demographic variables. These were performed either in line with widely used and defined demographic or clinical cut-offs, or with justified threshold levels. This was performed for patient age by 5-year banding, BMI by 5-unit banding, BEI for 5-unit banding, alcohol use by current NHS recommendations (no intake; up to 14 units per week; 14-28 units per week; and 28 units per week or more), smoking history, and peak previous activity level by modified Tegner Activity Scale categories. This data binning facilitated straight forward analysis, particularly for the ordinal categories.

For demographic data variables, the main categorical variables in the cohort were defined as sex, pre-op age group, pre-op BMI group pre-op, pre-op BEI group, Kellgren and Lawrence osteoarthritic score of op-knee, alcohol use group, smoking history, Scottish Index of Multiple Deprivation (SIMD) quintile, occupation category, peak activity level, comorbidity of diabetes, comorbidity of hypertension, long term use of paracetamol, long term use of NSAIDs, long term use of opiates, and whether the patient had previously undergone contralateral knee arthroplasty.

Demographic and clinical baseline factor correlations

To assess for variable collinearity, and potential statistical bias, the demographic and clinical baseline factors were initially compared using correlation coefficients. Due to the categorical nature of many of the variables, Spearman Rho methodology was used. Some statistically significant weak correlations were observed, alongside two strong correlations, and one moderate correlation. The full correlation matrix can be found in Appendix C: Additional Data.

Patient sex was found to strongly correlate with bioelectrical impedance, where the established biological propensity to higher body fat in females influenced results leading to higher BEI values ($r = 0.65$). Additionally, a moderate correlation was observed between sex and peak physical activity in life assessed by the modified Tegner Activity Scale ($r = 0.47$). Males were more likely than females to record a high score with this variable. Lastly, a strong correlation was observed between BMI and BEI data ($r = 0.64$), logically present due to the likelihood of higher body fat in those of shorter stature with higher body weight. For context, only a non-significant, very weak correlation was observed between sex and BMI ($r = 0.10$). Significance determines the likelihood that a result was found by chance when performing a statistical test. A probability-value (p-value) set at a level of 0.05 to determine a significant finding indicates that there is a 1 in 20 chance of the result being due to chance, and therefore a false positive.

A further comparison of Variation Inflation Factors (VIFs) was made between the four discussed variables to check further for covariation. VIFs assesses collinearity which can artificially overfit a regression modelling result to indicate a stronger relationship is present when it is not. All were found to be below 2, a low value, which allayed concern for mutual inclusion in subsequent multivariate analyses.

VIF evaluation was subsequently performed within all multiple variable models to assess for collinearity and to prevent potential bias and model overfit.

Cohort data contained over 180 categories of data per patient. This only encompassed summary PROMs and functional scores, with the number of categories much higher including raw data. To allow structured statistical analysis, definitive data variables were selected.

All data were combined from sub-cohorts for analysis. However, some categories were only obtained from a single sub-cohort by design. The cohort demographic similarities allowed reasonable comparison across the entire study cohort.

MAKRO Study Patient Data

The patient data results from the longitudinal cohort study provided insight into temporal trends of the patients early functional outcome metrics. The relationship between the metrics could be examined, and the time-points which showed greatest metric variance were selected for further investigation.

The interrogation of study results sought to answer the following research questions:

- How do functional outcome metrics change over time during early surgical recovery following total knee arthroplasty?
- Do different functional outcome metrics change at different rates post-TKA?
- Which outcome time-points during early recovery post-TKA are best to investigate the associated effects relating to patient background and baseline muscle physiology?

Biometric, Functional and Patient Reported Outcomes Data

Evaluating and understanding the temporal trajectory of each functional outcomes metric recorded provided information as to the recovery efficacy of the cohort. Additionally, it highlighted measurements which significantly changed within the duration of the study. The following research question was therefore addressed:

- How do functional outcome metrics change over time during early surgical recovery following total knee arthroplasty?

Summary graphical figures are displayed in-line for each metric's results with figures and tables. Supplementary figures are located in appendices where stated in Appendix C: Additional Data. Time-points are summarised in figure captions as weeks following surgery or specifically as follows: pre-assessment clinic (PAC), 6-weeks post-op (6W), 12-weeks post-op (12W), 6-months post-op (6M), and 12-months post-op (12M).

Patient biometric data, functional data and PROMs data from the MAKRO cohort study are displayed in Table 20. Study temporal data are displayed in probability-tables to show significant changes per metric over time in Table 21 and as graphical figures with 95% confidence intervals in Figure 30. Data for categories only measured in the Enhanced sub-cohort show lower numbers compared to those from the full cohort, therefore some response numbers varied by time-point.

Table 20 - Biometric, functional and patient reported outcomes summary results.

		Time-point														
		PAC			6 weeks post-op			12 weeks post-op			6 months post-op			12 months post-op		
Data Grouping	**Variable**	**n**	**m**	**StDev**	**n**	**m**	**StDev**	**n**	**m**	**StDev**	**n**	**m**	**StDev**	**n**	**m**	**StDev**
Biometrics	BMI	63	32.4	5.8	39	31.3	5.3	28	30.9	4.8	29	31.6	5.2	35	32.1	5.8
	BEI	56	38.0	8.9	35	36.3	7.9	25	36.1	6.3	27	36.6	6.8	31	35.6	7.8
	Op Knee ROM	59	96.4	12.7	42	82.9	18.8	29	90.2	18.9	29	98.7	17.1	36	99.3	20.6
	Average Heart Rate (bpm)	27	67.6	7.9	29	70.0	8.3	24	68.3	9.2	26	69.4	10.2	24	67.2	10.4
	Average Sleep Duration (mins)	27	07:51	01:16	29	07:54	01:33	24	07:51	00:53	26	07:43	00:47	25	07:37	01:14
	Light:Deep Sleep Ratio	27	1.5	0.6	29	1.7	0.9	24	1.5	0.7	26	1.4	0.6	25	1.4	0.8
Direct Functional Assessment	NLR Max Power Controlled by Body Weight	57	0.5	0.4	39	0.7	0.4	28	1.0	0.5	28	1.2	0.4	35	1.3	0.6
	ALF Score	56	30.8	14.7	40	33.3	17.0	28	26.5	14.5	29	22.8	8.7	35	23.9	10.3
	Daily Step Count	27	3698.9	2189.7	29	2960.5	2249.5	24	3743.4	2239.1	26	4723.0	2086.2	25	4629.7	2731.8
PROMs	EQ-5D Index	60	0.4	0.3	56	0.6	0.3	48	0.7	0.2	44	0.8	0.2	44	0.8	0.2
	EQ-5D Health VAS	60	70.6	19.3	56	75.9	18.6	47	78.6	18.4	44	83.5	15.3	43	79.6	18.8
	EQ-5D Pain VAS	62	50.8	22.3	56	59.2	26.9	48	67.9	30.3	44	77.5	24.0	42	78.5	25.6
	FJS Score	61	11.5	13.8	56	28.4	27.1	48	41.9	29.5	43	51.1	25.8	44	56.7	28.4
	KOOS Symptoms Dimension	59	41.7	18.4	55	61.2	20.1	47	69.8	18.7	47	72.5	19.1	47	77.9	19.4
	KOOS Pain Dimension	58	38.6	17.1	54	61.9	20.6	48	71.1	19.2	47	73.9	19.1	48	78.2	21.0
	KOOS ADL Dimension	60	44.3	17.9	54	66.1	19.9	48	73.9	18.7	47	76.1	18.7	48	79.1	20.5
	KOOS Sport and Rec. Dimension	60	14.3	20.2	52	21.3	26.5	47	33.8	33.4	47	38.1	31.2	47	37.9	32.7
	KOOS QOL Dimension	60	22.4	17.4	52	51.3	24.6	47	58.6	24.3	47	65.4	23.9	47	67.3	27.8
	KOOS5 Composite Index	60	32.3	14.0	55	52.7	18.7	48	61.5	18.0	47	65.2	19.0	48	68.1	20.1
	Oxford Knee Score	62	21.3	8.2	56	27.9	9.5	48	34.9	8.2	44	37.5	8.2	44	37.9	8.4

Table 21 – Probability tables showing statistical differences in metric between study time-point for patient biometric functional and PROMs data. Comparisons performed with omnibus One-way ANOVAs and, if statistically significantly different (alpha 0.05), interrogated with inter-time-point post-hoc pairwise comparison using t-tests with pooled standard deviations and Bonferroni-Holm (Holm) corrections for multiple comparisons. Sample n shown at sub-table base.

A

Range of Movement (operated knee)

Omnibus ANOVA p = 0.000

	PAC	6W	12W	6M	12M
6W	0.002	-	-	-	-
12W	0.497	0.433	-	-	-
6M	1.000	0.002	0.397	-	-
12M	1.000	0.001	0.282	1.000	-
n	59	42	29	29	36

B

Leg Extensor Power (power/weight ratio)

Omnibus ANOVA p = 0.000

	PAC	6W	12W	6M	12M
6W	0.221	-	-	-	-
12W	0.000	0.073	-	-	-
6M	0.000	0.000	0.252	-	-
12M	0.000	0.000	0.104	0.534	-
n	57	39	28	28	35

C

Aggregated Locomotor Function Score

Omnibus ANOVA p = 0.005

	PAC	6W	12W	6M	12M
6W	1.000	-	-	-	-
12W	0.891	0.289	-	-	-
6M	0.100	0.023	1.000	-	-
12M	0.154	0.036	1.000	1.000	-
n	56	40	28	29	35

D

Daily Step Count

Omnibus ANOVA p = 0.038

	PAC	6W	12W	6M	12M
6W	1.000	-	-	-	-
12W	1.000	1.000	-	-	-
6M	0.923	0.063	1.000	-	-
12M	1.000	0.093	1.000	1.000	-
n	27	29	24	26	25

E

EQ-5D Index

Omnibus ANOVA p = 0.000

	PAC	6W	12W	6M	12M
6W	0.000	-	-	-	-
12W	0.000	0.187	-	-	-
6M	0.000	0.006	0.401	-	-
12M	0.000	0.000	0.187	0.485	-
n	60	56	48	44	44

F

EQ-5D Health VAS

Omnibus ANOVA p = 0.008

	PAC	6W	12W	6M	12M
6W	0.725	-	-	-	-
12W	0.215	1.000	-	-	-
6M	0.005	0.292	1.000	-	-
12M	0.131	1.000	1.000	1.000	-
n	60	56	47	44	43

G

EQ-5D Pain VAS

Omnibus ANOVA p = 0.000

	PAC	6W	12W	6M	12M
6W	0.318	-	-	-	-
12W	0.004	0.318	-	-	-
6M	0.000	0.004	0.318	-	-
12M	0.000	0.003	0.281	0.861	-
n	62	56	48	44	42

H

Forgotten Joint Score

Omnibus ANOVA p = 0.000

	PAC	6W	12W	6M	12M
6W	0.002	-	-	-	-
12W	0.000	0.022	-	-	-
6M	0.000	0.000	0.169	-	-
12M	0.000	0.000	0.022	0.304	-
n	61	56	48	43	44

I

KOOS - Symptoms Dimension

Omnibus ANOVA p = 0.000

	PAC	6W	12W	6M	12M
6W	0.000	-	-	-	-
12W	0.000	0.100	-	-	-
6M	0.000	0.017	0.505	-	-
12M	0.000	0.000	0.133	0.355	-
n	59	55	47	47	47

J

KOOS - Pain Dimension

Omnibus ANOVA p = 0.000

	PAC	6W	12W	6M	12M
6W	0.000	-	-	-	-
12W	0.000	0.078	-	-	-
6M	0.000	0.012	0.583	-	-
12M	0.000	0.000	0.228	0.583	-
n	58	54	48	47	48

K

KOOS - ADLs Dimension

Omnibus ANOVA p = 0.000

	PAC	6W	12W	6M	12M
6W	0.000	-	-	-	-
12W	0.000	0.171	-	-	-
6M	0.000	0.048	0.901	-	-
12M	0.000	0.005	0.555	0.901	-
n	60	54	48	47	48

L

KOOS - Sport & Recreation Dimension

Omnibus ANOVA p = 0.000

	PAC	6W	12W	6M	12M
6W	0.843	-	-	-	-
12W	0.005	0.163	-	-	-
6M	0.000	0.031	1.000	-	-
12M	0.000	0.031	1.000	1.000	-
n	60	52	47	47	47

M

KOOS - Quality of Life Dimension

Omnibus ANOVA p = 0.000

	PAC	6W	12W	6M	12M
6W	0.000	-	-	-	-
12W	0.000	0.383	-	-	-
6M	0.000	0.018	0.383	-	-
12M	0.000	0.006	0.319	0.705	-
n	60	52	47	47	47

N

KOOS5 Index Score

Omnibus ANOVA p = 0.000

	PAC	6W	12W	6M	12M
6W	0.000	-	-	-	-
12W	0.000	0.055	-	-	-
6M	0.000	0.003	0.648	-	-
12M	0.000	0.000	0.230	0.648	-
n	60	55	48	47	48

O

Oxford Knee Score

Omnibus ANOVA p = 0.000

	PAC	6W	12W	6M	12M
6W	0.000	-	-	-	-
12W	0.000	0.000	-	-	-
6M	0.000	0.000	0.325	-	-
12M	0.000	0.000	0.298	0.805	-
n	62	56	48	44	44

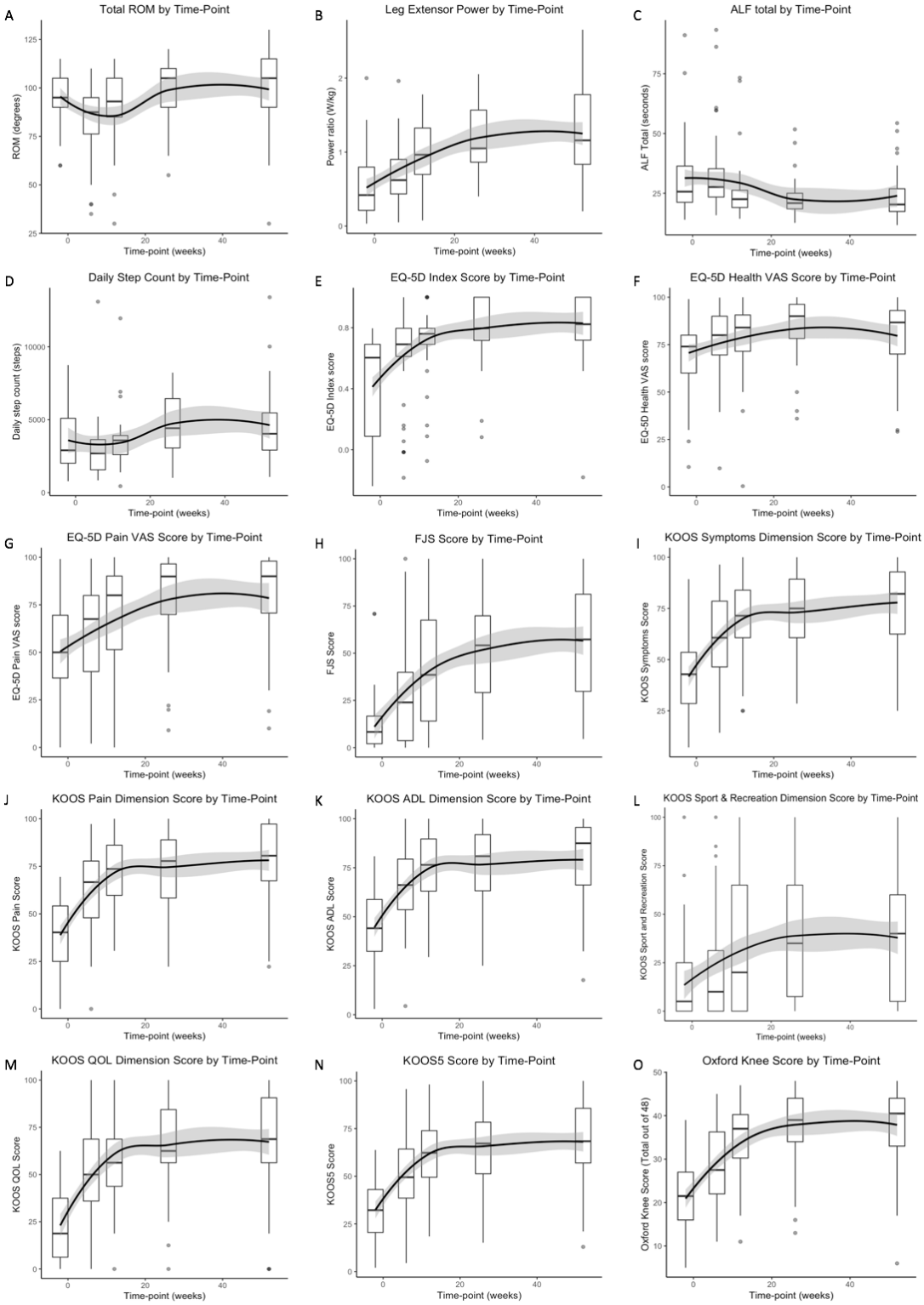

Figure 30 – Time-course data series for biometrics, functional assessments, and PROMs scores with individual dimension representations. Dual boxplot and Loess (local regression) smooth fit curves in figures to show trends

over study time-course. Shaded areas represent 95% confidence intervals. Week zero represents TKA intervention. Time-point sample numbers listed in Table 20 and

Table 21.

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Patient Biometrics

Participant BMI and BEI values were relatively high across all time-points, with average BMI values classified as obese across all time-points. A ceiling effect was present for BEI values over 50% due to measurement device limitations discovered during the study. This affected 10% of baseline measurements. As described in the literature due to higher propensity to body fat, higher BEI values were observed in female participants compared to males across all time-points. This was not present for BMI values.

BMI was found to be similar across all time-points ($p=0.77$). A similar trend was also found with BEI measurements ($p=0.66$).

Patient op-knee range of motion showed variation between patients but typically improved following TKA. Patient Range of Movement (ROM) in the operated knee was found to be significantly different between time-points and was investigated further to determine where differences occurred during the study. Patient ROM initially decreased following their operation but had improved by 6-months post-op. No further ROM change was observed by 12-months post-op.

Directly Measured Functional Outcomes

During the study, the rate of change in ability of the control leg was noted in the majority of patients. Some had bilateral pain at their pre-op time-point, and some developed contralateral pain throughout the duration of the study. This pain reflected elements of their effort guarding while performing assessments, particularly the leg extensor power assessment. As such, this confirmed the selection of normalised power to body weight ratio over normalisation against control leg data. This body weight normalisation, controls for sex and patient stature (291). This created a constant which was reproducible by patient and time-point to allow sequential comparisons.

While pain was clearly present for the operative knee both in the preoperative assessment due to osteoarthritic pain, and then in the early post-operative window due to operative tissue damage, this was a constant across the entire patient cohort.

Patient leg extensor power to weight ratio was found to differ significantly across the study, with similar values observed 6-weeks following surgery but with improved values achieved by 12-weeks post-op. The values then remained similar for the remainder of the study (Table 21).

Patients' Aggregated Locomotor Function (ALF) score was found to differ over time (Table 21). A lower ALF score reflected a quicker performance following an intervention and therefore a combination of relative comfort, agility and confidence in locomotion. Changes were observed between 6-weeks and 12-weeks following surgery which then stabilised. The total time taken to perform the functional activity battery ranged from 93.34 seconds, observed in one patient at 6-weeks post-op, to 11.68 seconds, observed in another at 12-months post-op. A cluster effect was observed between 20 and 30 seconds, with patients scoring higher or lower noticeably exhibiting phenotypic functional issues or functional agility respectively. This indicated a potential sensitivity measurement range aspect of the functional assessment.

The average daily step count of participants was calculated as a mean from the exported 4-day raw data from Xaomi Mi Band activity monitors provided to those allocated to the Enhanced sub-cohort. Cohort mean step count reduced from 3850 to 2960 steps by 6-weeks following surgery, which then increased to 4700 steps by 6-months and remained stable. All values are lower than recommended health targets (238) but do show a 22% primary outcome improvement over baseline when examined in this way.

The average daily step count was found to be significantly different over time (Table 21), however, when investigated using pairwise comparisons with corrections for multiple comparisons, no time-point changes were deemed different with the stated alpha. Similar values were observed between pre-op, 6-weeks, and 12-weeks post-

op (p=1.00). The difference between 6-weeks and 6-months following surgery showed the largest non-significant different (p=0.06), which remained similar by 12-months (p=1.00).

The results for the average heart rate (p=0.78) and average nightly sleep duration (p=0.91), tested within the Enhanced cohort using the activity monitors, were found to be statistically similar across all time-points.

Patient Reported Outcome Measures

Patient EQ-5D index scores differed across time-points and were found to show the greatest improvements between pre-assessment and 6-weeks post-op. Further changes were observed by 6-months post-op which remained unchanged 6 months later (p=0.49).

The EQ-5D's Health VAS was found to change over time. Improvement was observed by 6-months post-op (mean change from 70.6±19.3 at PAC to 83.5±15.3 at 6 months, p=0.005). The Pain VAS also changed over time but showed positive changes earlier at 12-weeks post-op (mean change 50.8±22.3 at PAC to 67.9±30.3 at 12 weeks, p=0.004). For both, no further changes were observed after 6-months post-op ($p \geq 0.861$).

FJS scores for the patient cohort positively changed over the course of the study, with step-wise significant changes observed from pre-op to 6-weeks post-op (p=0.002) and again from 6-weeks- to 12-weeks post-op (p=0.022), after which scores plateaued ($p \geq 0.555$).

The Knee Injury and Osteoarthritis Outcome Score was analysed in its five separate dimension sub-scores and subsequently in its single index form. All six of the score totals were found to vary across the study time-points, however they showed differing rates of improvement. 5 of 6 of the KOOS scores initially improved by 6-weeks after surgery ($p<0.001$), with the sport and recreation dimension initially continuing to show similar values (p=0.843). The symptoms and pain dimensions sub-

scores improved at 12-weeks post-op to levels not seen until 6-months post-op in the activities of daily living, sport and recreation, and the quality of life sub-scores. The combined index score showed that all improvement had been achieved by 12-weeks post-op with no subsequent score increase ($p \geq 0.230$).

The Oxford Knee Score was found to differ across time-points, specifically between pre-op and 6-weeks post-op (21.3 ± 8.2 to 27.9 ± 9.5, $p<0.001$), and then again to its maximum level by 12-weeks post-op (34.9 ± 8.2, $p<0.001$).

Discussion of Univariate Biometric, Functional, and PROMs results

Patient Biometrics

Patient knee ROM has a threshold which dictates a combination of factors. A knee fixed in flexion and unable to extend fully can prove a tiresome burden due to constant muscular effort, and a knee lacking flexion past 80-90 degrees can be a large obstacle to performing ADLs such as climbing stairs, rising from a chair, and using a motor vehicle. With a larger total ROM, the likelihood of full extension and flexion past the stated threshold is increased and therefore reflected in the functional benefits of doing so. These factors were subsequently used as categorical variables during further tertiary analyses.

Directly Measured Functional Outcomes

While the four-day average step count allowed for snapshot data per time-point, the exact timing of the clinics may have influenced the activity of the patients. Research clinic assessments were scheduled on Wednesdays and Thursdays defined by clinic access schedule meaning that recording of activity occurred from day-of-clinic through to a Sunday or Monday night. While the recording period was thus standardised to include the weekend, the difference between weekday and weekend may have influenced the activity level either positively or negatively. Additionally, some clinic appointments occurred just prior to personal or national holidays, such as sedentary beach and sun-based vacations as well as family gatherings in late December, which would have provided uncharacteristic activity levels when compared to normal habits. The structure of the clinics didn't allow for flexibility here, so all results are taken as representative.

From discussions during clinic visits with patients, it became apparent that an element of habitual activity may have been observed during latter time-points. A patient may have possessed the ability to perform more steps but had no reason to do so. Once they had achieved their goal of reaching a local destination such as a shop, or a friend or family member's residence, they had no reason to venture

further. A comparison of pre-operative daily step counts to those at 6-months and 12-months post-op are displayed in Figure 31.

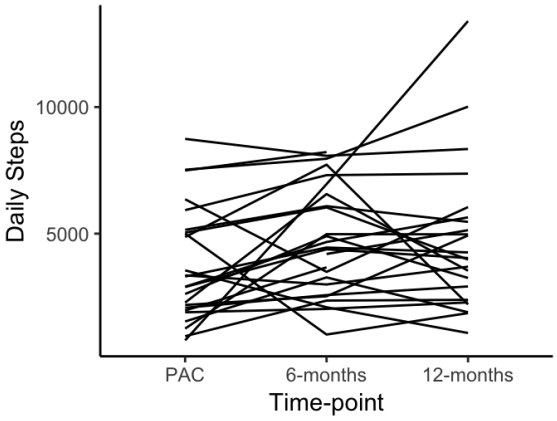

Figure 31 - Individual plots of average daily step count recorded following the preoperative assessment clinic, the 6-months post-op assessment, and the 12-months post-op assessment.

A small number of time-point step-count outliers were present within the cohort. The most apparent was a participant's change from roughly 800 daily steps pre-op to more than 13,000 steps 12 months following TKA, which was related to a return to manual labour work. Conversely, all major step-count reductions, such as a drop to zero, were related to other pathology.

Several patients commented that while they had accomplished roughly the same distance as at 6-months post-op, by 12-months post-op they were doing so at a faster walking pace. As the factor of walking speed or cadence was frequently mentioned by patients is likely to be a relevant factor across all time-points.

Investigations into sleep quality, for example determined through the extent of restlessness, disruptions or irregular circadian rhythms, may provide future insight into aspects of sleep disruption due to pain or other factors. However, the use of analgesic pharmaceuticals may counter this effect.

Within the aggregated locomotor function data, comprising a timed-up-and-go with a stair-set climb and descend, and an 8-meter walk, the steep stair descent component proved the greatest obstacle to some study participants. For those experiencing functional difficulty, the activity of steep stair descent with the weight-loaded knee in a compromised position would lead to them descending one stair at

a time, whilst favouring the non-operated knee, and using the bannisters to support themselves. This was likely to be due to the greater flexion required when descending stairs and the reduction in strength that this produces which subsequently accentuated any strength deficits that they had (392). This added the most time to their scores compared to their performance in the other areas of the test battery.

Patient Reported Outcome Measures

Overall, the study PROM data were in in-line with established databases of post-TKA responses which validated the results (Table 22). These are compared to local studies with similar case-mix (166,393), and to larger databases collected throughout the UK and Europe (394,395).

Table 22 - Comparative headline PROMs data from study and published databases with time-points. Hamilton and Giesinger collected data at 12-months post-TKA, Baker collated data collected between 6-months and 12-month post-TKA.

	Data (Mean (StDev))				
Category	Study data	Hamilton 2015	Giesinger 2015	Hamilton 2012	Baker 2012
OKS Pre-op	21.3 (8.2)	19.7 (7.4)	-	19.1 (7.5)	18.9 (7.8)
OKS Post-op (6-12M)	37.9 (8.4)	37.8 (7.9)	-	37.8 (7.9)	34 (11.7)
EQ-5D Pre-op	0.40 (0.30)	-	-	-	0.41 (0.27)
EQ-5D Post-op (6-12M)	0.80 (0.20)	-	0.85 (0.21)	-	0.71 (0.27)

The lower post-operative scores from the Baker et al. study likely reflects the different data collection time-window which included responses between 6-months post-op and 12-months post-op with responses from over 23000 patients.

Within the study data, the differences between the PROM tools' scores reflects the slight variances in their focuses. Particularly within the EQ-5D VAS scores, this could implicate the perceived value that pain contributes to overall health state. While pain had reduced, overall perceived health improvement was not seen for a further 3 months which reflects the diverse contributions to the overall health state.

Notable in the KOOS results was the sports and recreation sub-scores low final outcome level compared to the other sub-scores. The nature of post-operative clinical advice provision and the mechanical limitations and of the knee endoprosthesis dissuaded activities such as running and jumping which contributed

to the sub-score. The lack of ability to perform these and similar activities dictated a low sub-score in this dimension.

The differently observed recovery trajectories within the KOOS reflected the nature of the TKA procedure and the different targets of the sub-scores. The operation intends to remove symptoms and associated pain, however the true benefits from the translational functional elements take longer to appear. The pain from the operative trauma has disappeared within 3-months following the operation but the full improved strength and endurance take longer to appear. These are reflected in the higher scores in those sub-scores within the latter time-points.

The study data showed that some functional outcomes changed over time during early surgical recovery in the study cohort. Other recorded metrics remained similar through this period, particularly biometric data.

These data were examined for their individual temporal trends during early surgical recovery.

Comparison of Measured Outcome Temporal Trends

Differences in Recovery Rates by Chosen Metric

Having evaluated the metric trends in isolation, next comparing them to the other study metrics provided answers to two of the study research questions:

- Do different functional outcome metrics change at different rates post-TKA?
- Which outcome time-points during early recovery post-TKA are best to investigate the associated effects relating to patient background and baseline muscle physiology?

The difference in measured recovery rate differed by chosen outcome measurement within the study population. This occurred across outcome metric categories as well as between similar tools. Some measurements show significant sustained improvement by 6-weeks post-operation, while others initially worsened before improvements were observed by 12-weeks or 6-months post-op. Notably the operated knee ROM and the daily step count were worse than pre-op by 6-weeks of recovery time (Table 23).

The significant improvements in PROMs scores by 6-weeks post-op contrasts sharply with patients' direct functional measurements scores. While the PROM questionnaires capture more than just functional aspects of recovery, notably pain levels and some basic flexibility aspects, functional outcomes factor highly into index score weighting.

Table 23 – Rate of variation of different outcomes by time-point post-operation. Arrows represent an improvement or worsening in metric trend compared the previous time-point. Asterisks denote statistical significance (alpha = 0.05). Colours are distinct for each arrow type to allow for ease of recognition.

	Time-point post-operation			
	6-weeks	12-weeks	6-months	12-months
Functional - Clinic Measured				
ROM	↓*	↑	↑	→
Power (leg extensor ratio)	→	↑	↑	→
ADL performance (ALF score)	→	↑	→	→
Functional - Community Measured				
Average daily step count	↓	→	→	→
Patient Reported				
EQ-5D Index	↑*	↑*	↑	→
EQ-5D Health VAS	→	→	↑	→
EQ-5D Pain VAS	→	↑	↑	→
FJS	↑*	↑*	↑	→
KOOS Symptoms D.	↑*	↑	→	→
KOOS Pain D.	↑*	↑	→	→
KOOS ADL D.	↑*	↑	→	→
KOOS Sport & Rec.D.	→	↑	→	→
KOOS QOL D.	↑*	↑	→	→
KOOS5 Index	↑*	↑	→	→
OKS	↑*	↑*	→	→

6-week recovery data show that patients think that they have much improved at this stage of recovery compared to pre-operation. When compared to the directly measured functional outcomes at this time-point, the measured aspects provide a different picture. Patient operated knee joint ROM and average daily step count had decreased, while patient strength and function had remained similar.

Subsequently, there is a division in the progression of outcome measurements. Some functional and patient reported metrics reach their maximum values by 12-weeks of recovery, however others continue to show improvement through until 6-months or even 12-months following the operation (Table 24). The variation in maximum progression time-point is distinct even within score sub-dimensions such as the KOOS dimensions.

Table 24 – Final time-point at which each outcome measurement subsequently plateaus and does not meaningfully improve. Improvement fit trend defined by local regression curve.

	Time-point post-operation			
	6-weeks	12-weeks	6-months	12-months
Functional - Clinic Measured				
ROM			●	
Power (leg extensor ratio)			●	
ADL performance (ALF score)		●		
Functional - Community Measured				
Average daily step count			●	
Patient Reported				
EQ-5D Index			●	
EQ-5D Health VAS	●			
EQ-5D Pain VAS			●	
FJS				●
KOOS Symptoms D.				●
KOOS Pain D.				●
KOOS ADL D.		●		
KOOS Sport & Rec.D.		●		
KOOS QOL D.			●	
KOOS5 Index			●	
OKS			●	

Compared to preoperative values, by 12 weeks post-op, patients had achieved 82% of final (12 months post-op) KOOS5 PROM score, 63% of final leg power, 63% of final ADL performance measured by ALF, and 5% of final daily step count. The values further increased by 6 months post-op with KOOS5 score increased to 92% of final score, leg power at 94% final, ADL at 116% final, and daily step count at 110% of final value. This is visualised in the time-course graphs displayed in Figure 30 earlier.

These findings highlighted the utility of using a combination of diverse metrics to evaluate patient functional outcome and showed that patient evaluated outcome alone may not fully represent early functional outcome. They also emphasise the critical nature of the follow up time points chosen for clinical trials.

Interestingly, while patients may have the physiological ability to extend their range and increase their step count based on improved strength and perceived ability, it seems that they choose not to do so based on established lifestyle and activity habits. The weaker correlation at 12 weeks between ALF and step count was notable as it may reflect the improvement in strength and ability to perform representative ADLs quickly when assessed in clinic, but that the endurance is still lacking for activities

such as walking distance. This endurance may have recovered by the 6-month time point resulting in the improved correlation with the other functional metrics.

While not directly measured in this study, complimentary step count data such as step cadence or speed may further elucidate the picture in comparing walking ability and endurance between pre- and post-op function. Movement quality may have improved substantially, though it seems distance has not. Different sensitivity to recovery was observed across the range of functional outcome measurements. This reinforces the importance of multiple tool use in the design of clinical studies (188), to reflect the differing aspects of function, and that careful analysis is required due to the variation in metrics at different time-points.

Patients are able to demonstrate greater functional ability in tests of maximal capacity following total knee arthroplasty, but they demonstrate habitual levels of activity, consistent with pre-operative values suggestion that activity behaviour modification may be required to utilise the physical benefit of knee arthroplasty.

Once the arthritic pain is removed by surgical intervention, a patient may not suddenly double their daily step count, even though they have the capacity to do so. Additionally, a time period is required for the unstructured training adaption to gain skeletal muscle endurance for those activities to feel habitual, non-tiring, and comparatively effortless following a long pre-operative period of disuse. However, the genotype and phenotype of the patient muscle is key to determining the physiological propensity for this to be able to happen. An understanding of patient baseline physiology is therefore necessary to help clarify the causative factors within the study cohort.

Definitive Variables and Time-points for Multivariate Modelling Analysis

Functional Variables for Multivariate Analysis

Functionally assessed outcomes were examined to choose best representative results for multiple analysis comparisons. The primary decision for these selections came from the OARSI metrics recommendations, as with the initial choice of metrics, and the percentage of study data capture also factored into the final selection.

The categories were also to be selected to avoid overfit which would have resulted from collinearity or including categories that measured very similar aspects of function with parallel results. The initial data categories were already selected to measure different aspects of function.

Patient functional variables were compared to examine correlations (Table 25). Full matrices are located in Appendix C: Additional Data. Pearson correlation coefficients of pooled results from all time-points showed moderate correlations between ROM to Leg Power ($r=0.464$; $p<0.001$) and ALF score ($r=-0.431$; $p<0.001$), but not to Daily Step Count. Leg power showed correlations with Daily Step Count ($r=0.291$; $p=0.001$) and ALF score ($r=-0.570$; $p<0.001$). ALF score also showed moderate correlations Daily Step Count ($r=-0.427$; $p<0.001$).

Table 25 - Pearson correlation coefficients showing associations between all functional metric results collated from all time-points.

	Total op-knee ROM	Leg Power Ratio	ALF Score
Leg Power Ratio	0.464		
	<0.000		
ALF Score	-0.431	-0.570	
	<0.000	<0.000	
Daily Step Count	0.093	0.291	-0.427
	0.291	0.001	<0.000
Cell Contents:	*Pearson correlation*		
	P-Value		

When the factors were investigated at individual time-points, the data provided a similar picture. Associations become notably stronger at latter time-points as patients recovered from TKA (Appendix C: Additional Data).

The associations broadly indicate that improved function in one parameter likely translates to improved ability in another. Daily step count and knee range of motion were least associated. Extreme restrictions in ROM (e.g. flexion block <80° and fixed flexion >10°) lead to impaired locomotion in some patient but other participants remained fairly active in the community, measured by step count, despite limitations in movement range.

The chosen categories for multivariate modelling analysis were directly measured functional power evaluated by Nottingham Leg Rig controlled by body weight, timed ADL surrogate performance by Aggregated Locomotor Function score, and average daily step count recorded by electronic activity monitor. These were termed the primary surgical outcome metrics and were therefore examined during multivariate modelling. Knee range of movement was not included as a primary functional outcome metric due to it being categorised for modelling purposes as a biometric measurement similar to BMI. Instead it was included as a predictive metric during modelling.

PROMs Variables for Multivariate Analysis

The patient reported factors similarly required representative selection for use in multiple analysis comparisons. While each PROM tool had a unique question set, length and focus, an evaluation of collinearity was performed to measure the scoring overlap.

PROM collinearity was assessed with a correlation matrix and a regression model to assess Variation Inflation Factors (VIF). Moderate correlation was observed in the VIF responses (VIF 3-5) and strong correlation coefficients were observed across all PROMs tools ($r>0.70$). These results indicated that a single PROM tool should be selected to prevent multicollinearity leading to model overfit.

With one generic health score and three knee specific scores, the different specificities were apparent in the correlations data. The knee scores correlated more strongly with each other than with the EQ5D Index score. Due to the nature of the insight desired from the PROM, a knee specific score was more valuable regarding TKA outcome. Additionally, the highest response rate was necessary to maximise model power.

The KOOS5 score was therefore chosen over the other PROMs scores due to its specificity in the TKA population and its response rate being highest amongst all other PROMs tools in the study. The KOOS tool was also the longest of all the study questionnaires and therefore was the most sensitive to variation in response.

The KOOS5 Index score was found to correlate strongly with the EQ-5D and very strongly with the FJS and OKS PROMs tools at each time-point (Appendix C: Additional Data), making it a highly representative candidate of all the PROM data collected throughout the study period.

Definitive time-points for multivariate analysis

Multivariate modelling comparisons required definitive time-points. A very early outcome and one representing final study endpoint were to be selected. 12-months post-op was chosen as primary study endpoint from the study outset due to the study duration. Patient functionally measured and patient reported data both displaying plateau effects by 12-months post-op.

The study data were evaluated using time-point data levels instead of inter-time-point variable change due to the large pre-op variation in functional performance and PROMs data. Both changes (delta) in metrics and absolute levels are routinely compared in the orthopaedic PROMs literature (396). Categorising a patient by their 12-month outcome scores was selected to reflect real-world function rather than to control these values by their performance pre-op, as the initial values were likely affected by a large number of additional factors. Similarly, to control the values by patients' 6-weeks data missed a key early window into their recovery where much recovery has taken place. Patients have uniform limited mobility in the first days following TKA, but some go on to achieve relative normality within 6 weeks while others remain disabled. The underlying causes are complex, with pain and control of pain playing a major role. For example, activity inhibiting pain varied, as did analgesic use as shown by patients' Oxford Knee Score and VAS responses, and discussion their about analgesic use in clinic. These factors are investigated further within the multiple comparisons.

Very early recovery outcome variation was defined as the 12-weeks post-op time-point. This time-point was identified as showing large outcome variation within the cohort and the chosen primary outcome metrics, which allowed for further investigation of how a variety of background and physiological factors may influence this early stage and the rate of recovery following TKA. The 12-week time-point was selected over the nearest alternative time-points of 6-weeks post-op and 6-months post-op due to the respective excluding factors of residual surgical pain and the plateauing of metrics. Due to the nature of the patient cohort stratification, any functional factors were investigated within the Enhanced sub-cohort at this stage, but PROMs data were obtained from the whole study cohort.

Patient Biometric Data and Functional Outcomes: Results and Discussion

Patient biometric data were compared with the main functional outcomes to examine further trends. This was performed using Pearson correlation coefficients. Additional data matrices are located in Appendix C: Additional Data.

Sleep

Total sleep duration, categorised sleep duration, and sleep quality measured from activity monitor light sleep to deep sleep ratio were compared to KOOS5 score, Leg Power Ratio, ALF score, and Step count at the defined very early (12 weeks post-op) and early (12 months post-op) TKA functional outcome assessments. Overview data for the metrics at 12-months post-op are tabled in Table 26, and data from 12-weeks can be found in Appendix C: Additional Data.

Table 26 - Correlation coefficients of sleep data from 12-months post-op compared to functional outcome data.

	KOOS5 Index (12M)	Leg Power Ratio (NLR 12M)	ALF Score (12M)	Daily Step Count (12M)
Total Sleep at 12 months post-op	-0.068	-0.249	0.223	-0.528
	0.748	0.230	0.284	0.007
Total Light Sleep at 12 month post-op	0.209	0.306	<0.001	-0.224
	0.316	0.137	0.998	0.281
Total Deep Sleep at 12 months post-op	0.219	0.063	-0.322	-0.208
	0.293	0.763	0.116	0.319
Total Light Sleep/ Total Deep Sleep at 12 months post-op	-0.235	-0.148	0.511	-0.131
	0.259	0.481	0.009	0.532

No significant correlations were identified between duration of light or deep sleep and functional outcomes at either time-point ($p>0.05$). Total sleep duration was correlated with ALF score at 12-weeks post-op ($r=0.45$; $p=0.029$), inferring longer sleep duration was associated with slower ALF performance during very early outcomes. Total sleep was also negatively correlated with daily step count at both 12-week ($r=-0.49$; $p=0.015$) and 12-month time-points ($r=-0.53$; $p=0.007$). This showed longer sleep was correlated with lower daily step count. The ratio of light sleep to deep sleep was correlated with a higher ALF score at 12-months post-op ($r=0.51$; $p=0.009$). This indicated a lack of deep sleep, or a disturbed and shallow sleep, being associated with slower times in ALF battery performance. An examination of pain scores showed no significant effect on the metric ($p>0.05$).

These preliminary results show sleep duration, depth and inferred quality provide a relevant metric to be examined in relation to patient function outcomes. Future examination of sleep factors in TKA outcomes with specialised PROM tools such as the Pittsburgh Sleep Quality Index (397) or Epworth scale (398) may identify clearer factors such as sleep deficiency, sleep latency, and the resulting psychometric and physiological effects (399).

Knee and Thigh Circumference

Knee and thigh circumferences were measured at 6-months post-op to explore aspects of Arthrogenic Muscle Inhibition (AMI), where local swelling can impair knee function by reducing the efficacy of innervation (Table 27).

Mean knee circumference was measured as larger in op-knee at 6-months post-op compared to contralateral knee but found to be statistically similar using paired t-tests (p=0.078). Thigh circumference was also but found to be statistically similar between sides (p=0.139).

Table 27 - Knee and thigh circumference measurement data taken at 6-months post-op in the Enhanced cohort.

Variable	n	m	StDev
Operated Knee Circumference (cm)	20	44.15	4.31
Contralateral Knee Circumference (cm)	20	43.05	3.64
Operated leg Mid-thigh Circumference (cm)	20	50.53	6.48
Contralateral leg Mid-thigh Circumference (cm)	20	49.38	5.49
Op. Knee Circ. / Conta. Knee Circ.	20	1.02	0.06
Op. Leg Mid-thigh Circ. / Contra. Leg Mid-thigh Circ.	20	1.02	0.06

Single time-point investigation of knee and thigh circumference with functional outcomes identified skeletal muscle bulk, continued knee swelling, and assessed differences between legs using Pearson correlation coefficients identified a single significant correlation between larger operated knee vs contralateral knee circumference as associated with longer ALF performance times (r=0.48; p=0.033) (Table 28).

Table 28 -Correlations between knee and thigh circumference measurements and functional outcomes data at 6-months post-op.

	KOOS5 Index (6M)	Leg Power Ratio (NLR 6M)	ALF Score (6M)	Daily Step Count (6M)
Operated Knee Circ. / Contra. Knee Circ.	-0.257	-0.198	0.477	-0.065
	0.274	0.417	0.033	0.797
Operated Knee Mid-thigh Circ. / Contra. Knee Mid-thigh Circ.	0.084	-0.244	0.042	-0.091
	0.726	0.314	0.859	0.720
Operated Knee Circ.	0.012	-0.151	0.096	0.247
	0.961	0.536	0.687	0.323
Operated Knee Mid-thigh Circ	0.054	-0.268	-0.024	0.102
	0.821	0.267	0.921	0.688
Contra. Knee Circ.	0.199	-0.042	-0.212	0.299
	0.401	0.865	0.369	0.229
Contra. Knee Mid-thigh Circ.	0.005	-0.163	-0.039	0.164
	0.984	0.504	0.869	0.514

Comparably larger prosthetic knee at 6-months post-op can be related to implant size as well as remaining swelling from surgical trauma. At 6-months post-op, concern about infection had been allayed, with a visual inspection of the knee during research clinics to confirm this. The physiological impact on the local tissue resulting from differential swelling likely include elements of arthrogenic muscle inhibition (AMI), which leads to functional impairment as assessed during ALF testing (126). Future examination with nerve conduction studies would elucidate this effect further.

Change in Activity Level

Patient reported activity level was assessed using the modified Tegner activity scale to determine previous peak activity, activity level at time of surgical pre-assessment, and at 12-month post TKA (Table 29).

Table 29 - Activity level data measured by modified Tegner Activity Level.

Variable	n	m	StDev
Life Peak Tegner	61	6.3	2.8
Full Cohort Tegner at PAC	60	1.3	0.8
Paired Cohort (to 12M data) Tegner at PAC	36	1.4	0.8
Tegner at 12-months post-TKA	36	2.0	1.3

Recreational activity level significantly improved by 12-months post-op (p=0.005). The mean activity level change from 1 to 2 represented an improvement from an ability to walk on even ground and any vocational work restricted to desk-work, to

an ability to walk sustained on uneven ground and participate in golf, bowls, curling or similar sporting activities, and to undertake vocational work involving light labour such as that required by a shop assistant or school teacher.

Examination of Pearson correlations between 12-month reported activity level and 12-month functional outcomes identified strong correlations with activity level and KOOS5 score (r=0.639; p<0.001), leg power (r=0.711; p<0.001), and ALF score (r=-0.754; p<0.001). Comparison with daily step count were non-significant (Table 30).

Table 30 – The correlations between final functional outcome metrics and return to activities in the community with Pearson correlation coefficient. Both r and p values are presented.

	KOOS5 Index (12M)	Leg Power Ratio (NLR 12M)	ALF Score (12M)	Daily Step Count (12M)
Activity level (Tegner) 12M	0.639	0.711	-0.754	0.306
	<0.001	<0.001	<0.001	0.137

Same time point general-to-specific predictive modelling was examined to determine the greatest contributions of clinic and self-reported tools influencing wider activity level as measured by Tegner (Table 31). The hierarchical investigation determined that clinic based ALF score was the greatest influence (p<0.01), with leg power also contributing (p=0.03). Combined they predicted 60% of reported Tegner score.

Table 31 - Primary functional outcome factors as model contributors to activity score (Tegner) 12 months post-op. The second model also includes pre-operative Tegner activity level as a predictive factor.

Response Metric	Ranked contributing factors (significance; coefficient (2 s.f.))	Contribution (R-Sq Adj)	Significance (degrees of freedom)
Activity Level (Tegner) 12 months following TKA	ALF Score at 12 months post-op (<0.01; -0.06) Leg Power controlled by body weight at 12 months post-op (0.03; 0.75)	0.60	<0.001 (32)
Activity Level (Tegner) 12 months following TKA	ALF Score at 12 months post-op (<0.01; -0.06) Activity Level (Tegner) pre-op (0.02; 0.40) Leg Power controlled by body weight at 12 months post-op (0.05; 0.66)	0.66	<0.001 (30)

Further examination of sex, age, peak life activity level, and analgesic use found no significant influences. Introducing the known pre-op activity level into the model, with a cohort real activity range varying from an inability to move around up to an ability to walk on a flat, even surface, provided another 6% of prediction to the model.

The results highlight two directly measured clinic-based outcomes that can strongly predict a return to greater levels of activity in the community at 1 year following TKA. Therefore, the targeting of post-operative rehabilitation to improve these functions should correlate with a more favourable achievement of desired activity levels. Furthermore, the lack of correlation with step count indicates additional evidence that total daily step count is likely habitual despite functional ability.

Analgesic Use

Analgesic medication is routinely used to combat musculoskeletal pain and its limitation of function. The comparison of patient managed analgesia and surgical outcomes can indicate if all patients with positive outcomes are pain free or if they achieve function despite continuing pain.

Table 32 -The response data of analgesic medication use pre-op and at 12-months post-op

Variable	Response n	% using
Paracetamol use pre-op	63	73.0
NSAID use pre-op	63	25.4
Opiate use pre-op	63	42.9
Paracetamol use 12-months post-op	36	33.3
NSAID use 12-months post-op	36	5.6
Opiate use 12-months post-op	36	25.0

Pre-op and final time-point (12-months post-op) data was collected on patient analgesia use in three categories: paracetamol, NSAIDs, and opiates (Table 32). This was recorded categorically. Use reductions were observed across all three categories, with the response rate for the latter time-point at 57%. Final time-point analgesic usage data and EQ-5D pain scores, to provide insight into pain levels, were then compared to functional outcomes using Pearson correlation coefficients (Table 33).

Table 33 – Correlation assessment between final functional outcome metrics and final time-point (12 months post-op) analgesia use and PROM pain score with Pearson correlation coefficient. Both r and p values are presented.

	KOOS5 Index (12M)	Leg Power Ratio (NLR) (12M)	ALF Score (12M)	Daily Step Count (12M)
Paracet. use 12 months post-op	-0.273	-0.152	0.245	0.284
	0.108	0.385	0.156	0.169
NSAIDs use 12 months post-op	0.039	-0.060	0.063	0.106
	0.824	0.730	0.721	0.614
Opiates use 12 months post-op	-0.130	-0.257	0.267	-0.178
	0.451	0.136	0.121	0.393
EQ5D Pain VAS 12M post-op	0.557	0.238	-0.426	-0.204
	0.000	0.231	0.027	0.402

No correlations were identified with analgesia use and functional outcome score. Lower pain level as measured by EQ-5D VAS was correlated with higher KOOS5 Score ($r=0.557$; $p<0.001$) and faster ALF performance ($r=-0.426$; $p=0.027$). The relationship between pain level and medication use at this time-point was also examined with Pearson correlation coefficients.

Table 34 - Correlation assessment between final functional outcome metrics and final time-point (12 months post-op) analgesia use with Pearson correlation coefficient. Both r and p values are presented.

	Paracet. use 12 months post-op	NSAIDs use 12 months post-op	Opiates use 12 months post-op
EQ5D Pain VAS 12M post-op	-0.666	0.082	-0.370
	0.000	0.684	0.057

No association was identified between pain score and NSAID use. Correlations were found between pain score and paracetamol use ($r=-0.666$; $p<0.001$) and opiate use ($r=-0.370$; $p=0.057$) (Table 34). Further investigation of the correlation with opiate use was made with general-to-specific regression modelling due to the p-value of the correlation being marginally above the 0.05 alpha. This was found to be a non-significant ($p=0.611$) association. The same methodology found paracetamol use alone predicted 42% of pain score at final time-point, with 38 points (EQ-5D Pain VAS score) higher pain for those making use of paracetamol ($p<0.001$).

The results show that while patients' use of some pain medication, namely paracetamol, strongly correlated with their reported pain levels, their use of opiates and particularly NSAIDs did not.

Knee ROM

As previously discussed, restricted knee joint range of movement can limit functional ability.

Patient ROM data was previously examined and shown to initially reduce following surgery and then significantly improve again by final time-point. Comparison of patient data on fixed flexion and flexion block prior to surgery and at final-time point can be compared to 12-month functional outcomes can identify relationships.

Limited range of movement was defined as inability to extend the leg less than 5 degrees from fully straightened, and as an inability to flex the knee past 90 degrees of flexion which corresponded with previously defined ROM limitations (400). Data were analysed using Pearson correlation coefficients (Table 35).

Table 35 - Range of motion limitations by pre-operative and final time-point.

Variable	Response n	% with ROM limitation
Flexion Block – PAC	58	10.3
Flexion Block – 12-months post-op	36	11.1
Fixed flexion – PAC	58	50.0
Fixed Flexion – 12 months post-op	36	33.3

A moderate and significant correlation was observed in those who had pre-op fixed flexion who still had fixed flexion 12-months following their operation (Table 36).

Table 36 - Correlation assessment between patients' pre-op and 12-month post-op knee range of movement limitations with Pearson correlation coefficient. Both r and p values are presented.

	Fixed Flexion Pre-Op	Fixed Flexion 12-months Post-Op	Flexion <90 degrees Pre-Op
Fixed Flexion 12-months Post-Op	0.354		
	0.034		
Flexion <90 degrees Pre-Op	0.000	0.213	
	1.000	0.212	
Flexion <90 degrees 12-months post-op	-0.177	0.313	0.213
	0.302	0.064	0.212

Table 37 - Correlation assessment between final functional outcome metrics and knee range of movement limitations with Pearson correlation coefficients. indicates that no participants of the enhanced cohort, who recording step count data, had pre-op flexion limited to 90 degrees or less from straight. Both r and p values are presented.*

	KOOS5 Index (12M)	Leg Power Ratio (NLR 12M)	ALF Score (12M)	Daily Step Count (12M)
Fixed Flexion Pre-Op	0.158	0.038	0.035	-0.180
	0.296	0.828	0.840	0.389
Fixed Flexion 12-months Post-Op	-0.368	-0.321	0.479	-0.183
	0.027	0.060	0.003	0.381
Flexion <90 degrees Pre-Op	-0.077	-0.321	0.193	*
	0.605	0.061	0.266	*
Flexion <90 degrees 12-months post-op	-0.485	-0.230	0.161	0.478
	0.003	0.184	0.356	0.016

No relationship was found between pre-op values and final outcomes. Weak and moderate correlations were identified between final study time-point ROM limitations and selected final functional outcomes (Table 37). A reduction in PROM scores was observed due to fixed flexion (r=-0.368; p=0.027) and flexion block (r=-0.485; p=0.003). ALF scores were significantly increased by fixed flexion (r=0.479; p=0.004). Increased step count was found to correlate with flexion block, however this was no longer present once outliers were removed. 3 participants of 25 who recorded 12-month step count data had limited flexion. Two of these participants were the only individuals to record above 10000 step per day.

Additionally, pre-op ROM limitations were found to significantly increase pre-op ALF timed score (r=0.28, p=0.03) and to reduce patient reported function (r=-0.38, p=0.03) (Table in Appendix C: Additional Data).

The negative impact of patient ROM issues on patient reported outcome at final time-point shows the negative effect of fixed flexion limitation on subjective symptoms, and on ADL function as measured by ALF score.

Patient ROM limitations show negative effects on concurrent final outcomes, with a fuller range of movement achieving close to straight extension and flexion past 90 degrees linked to better functional outcomes at 12-month following TKA.

Chapter 5 Summary

This chapter identified the recruitment and data collection flow of the MAKRO study, including its correspondence with expected demographic and functional composition as compared to previous research and national databases. Demographic factors were also identified for subsequent use in multivariate analyses.

Most outcome metrics were shown to change over time, with the notable exception of step count activity which indicated a return to pre-operative habitual activity level by 12-months post-TKA. Patient PROMs and direct functional assessments recovered at different rates following TKA and showed maximal level plateauing at different postoperative time-points. Moderate correlations were observed between direct functional assessments and strong correlations were observed between PROM tools. As such, multiple direct measurements and a single PROM tool were selected for use in multivariate analyses. The 12-weeks and 12-months postoperative time-points showed the greatest data variation and therefore possibility for differential insight based upon patient background and baseline muscle physiology.

The effect of sleep, arthrogenic muscle inhibition, and knee range of movement limitations were examined in relation to functional performance. The relationship between analgesic use, activity levels and function were also explored, with suggestions that targeted rehabilitation maximising ALF-related ADL performance and leg extensor power lead to maximum return to activity post-TKA.

Chapter 6: Results - Laboratory Patient Physiological Data

Laboratory data are presented separately in their respective methodology before they are analysed together. Histological results are followed by molecular results. This section aims to answer the following research questions:

- What is the variation in patient muscle physiological factors at time of surgery?
- How do patient muscle physiological factors at time of surgery relate to each other?

Samples were collected from 58 study participants and were all examined using the defined laboratory methodology. See CONSORT-style study flow charts for detail (Figure 27).

Immunofluorescence

Final summary data were collated from the replicates to provide single continuous variables in each measurement category. As a result, each sample and therefore participant had a measured number of type 1 and type 2 skeletal muscle fibres, and also a measurement of the mean lesser diameter per fibre type.

Skeletal Muscle Fibre Types and Fibre Size

Skeletal muscle fibre type data showed a larger number of type 2 fibres than type 1 fibres ($p<0.001$) with a mean ratio of Type 1:Type 2 of 0.70 ± 0.29, providing a mean vastus medialis fibre type distribution of 41% type 1 fibres and 59% type 2 fibres (Table 38).

Table 38 - Histological data for study skeletal muscle biopsies.

Variable	N	m	StDev
Number of Type 1 skeletal muscle fibres	58	20.78	7.44
Number of Type 2 skeletal muscle fibres	58	32.28	10.72
Ratio of Type 1/Type 2 skeletal muscle fibres	58	0.70	0.29
Lesser diameter of Type 1 skeletal muscle fibres (µm)	58	60.27	11.45
Lesser diameter of Type 2 skeletal muscle fibres (µm)	58	60.32	11.95

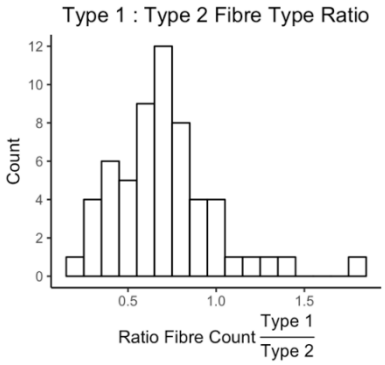

Figure 32 - Histogram count (bin-width 0.1) for skeletal muscle fibre type ratio. Mean ratio value 0.70 (41% type 1, 59% type 2). n=58.

Study results correspond to the literature for fibre type proportions within the quadriceps muscle group (401). Similarly, in correspondence with previous findings, a higher percentage of type 2 fibres were found in males (60%) compared to females (58%) (402). These findings can differ depending on the biopsy depth within a muscle group (401), but the parametric nature of the data likely show that the adherence to the sampling procedure instructions was sufficient standardisation (Figure 32). Further demographic comparative investigations are present later in this results section.

Fibre lesser diameter measurements were identified as marginally larger in Type 2 fibres but were found to be statistically similar when assessed with paired t-tests (p=0.962).Both fibre types showed a normal distribution amongst the population (Figure 34).

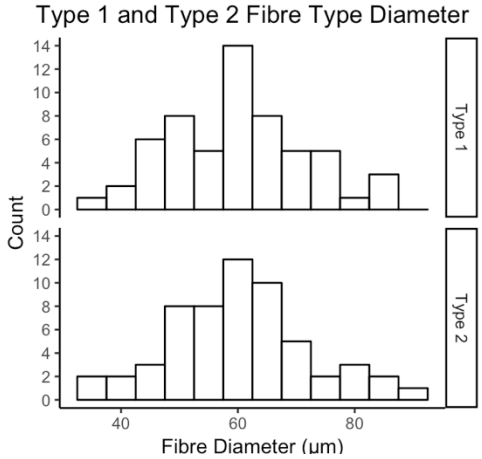

Figure 34 - Histogram distribution (bin-width 5μm) of fibre type diameters by fibre type. Mean values: Type 1 – 60.27μm, Type 2 – 60.32μm. n=58.

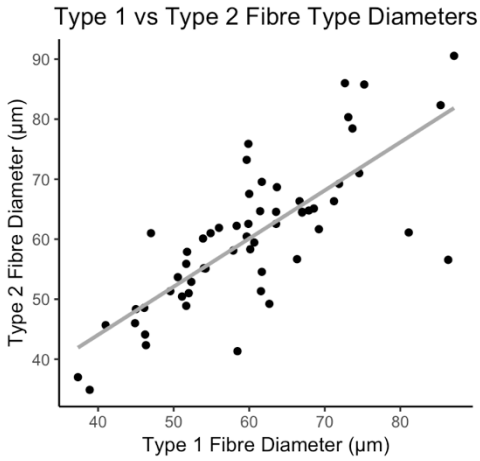

Figure 33 - Type 1 versus type 2 skeletal muscle fibre diameter. y = 15.79 + 0.74 · x, R^2(adj)=0.58, n=58.

Mean diameter histological results are similar to previous findings for this muscle group in the literature, though here performed in a comparably older population (402).

The relationship between fibre diameters was found to strongly correlate when examined with Pearson correlation coefficents (r=0.769; p<0.001). When data were displayed by scatterplot, the larger overall average diameter of the type 2 fibres was represented in the linear regression equation y-axis-intercept (+15.8) (Figure 33).

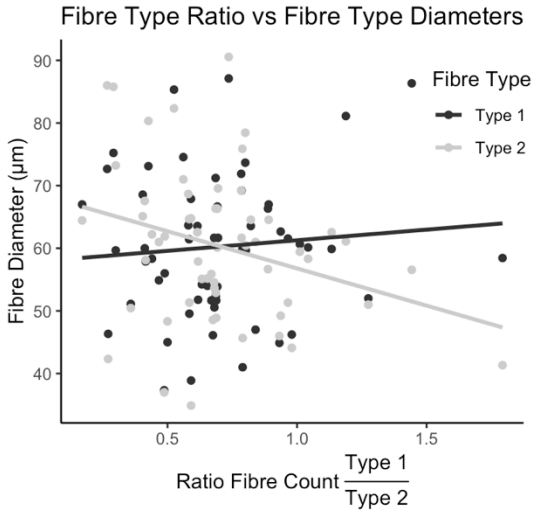

Figure 35 – Individual fibre diameter compared to individual fibre type count ratios. Type 1 fibres showed no relationship with ratio (R^2(adj)=0.02). Type 2 fibres showed a weak relationship with ratio (R^2(adj)=0.10). By the nature of the ratio metric, diameter values from single individuals share x coordinates.

A comparison of fibre type diameter to count ratios found that type 2 diameter weakly influenced the likely ratio, while type 1 fibre diameter had less bearing on the relative number observed (Figure 35). This is likely due to different underlying stimuli behind isolated fibre type hypertrophy. While there were a higher overall percentage of type 2 fibres observed per individual, the fibre diameters were not equally correlated to the number of fibres observed.

Using Pearson correlation coefficients, a weak correlation of fibre type diameter was found with sex ($r=0.354$; $p<0.001$), with male participants showing larger diameters. Older individuals possessed smaller fibre diameters ($r=-0.312$; $p=0.001$). This was more pronounced in type 2 ($r=-0.369$; $p=0.004$) compared to in type 1 fibres ($r=-0.252$; $p=0.056$). No correlation was identified between fibre count ratio and sex ($p=0.516$) or with age ($p=0.099$) The findings regarding sex and age correlate with previous trends in the literature (42,43,402).

Similarly analysed, no association was identified between fibre type proportion ($r=-0.151$; $p=0.305$) or type 2 fibre diameter data ($r=0.170$; $p=0.249$) and fixed flexion ROM block. However, a weak correlation was identified between type 1 fibre diameter and fixed flexion ($r=0.291$; p0.045) showing an association between an inability to fully straighten the knee joint and a larger diameter of type 1 skeletal muscle fibres in vastus medialis.

Real-time polymerase chain reaction (qPCR) – Relative Gene Expressions

Molecular Results Quality Steps

Molecular target gene relative expression values (ΔC_q) were calculated in line with the described protocol providing results for each target gene per patient. Molecular data sample numbers varied per gene of interest (GOI) due to the multiple quality checks on the raw data (Table 39).

Table 39 - Sample numbers for qPCR method positive relative expression level data. HK = housekeeping reference genes. GOI = Gene of interest. Some exclusion categories overlap due to quality control steps. The final n for each positively expressed gene target should be noted as the "n Included" row, which is highlighted.

GOI:	MyoD1	Pax7	Myf5	Myog	CDKN2A	IL6	TNF
n patients	58	58	58	58	58	58	58
n excluded HK	9	9	9	9	9	9	9
n GOI negative	6	0	0	0	39	25	10
n GOI excluded	19	11	43	20	3	12	17
n Included	**34**	**46**	**16**	**39**	**8**	**16**	**20**

Nine samples were excluded from all target genes of interest (GOI) analysis due to housekeeping gene data resulting in an inability to normalise the expression data. The housekeeping exclusions were due to negative findings, exclusions based upon melt curve anomalies indicating incorrect product amplification, and from replicate exclusions. Each housekeeping gene required two out of three replicates to be consistent to be included, and each sample required a minimum of two included housekeeping genes to calculate a housekeeping mean to be used to calculate ΔC_q.

The myogenesis GOI panel (MyoD1, Pax7, Myf5, and Myog) had low to no exclusion due to negative expression. This was only observed with MyoD1 in 6 samples. The inflammatory and senescence GOI panels showed higher exclusion due to no identified expression. No expression was found in 39 samples of CDKN2A, 25 samples of IL6, and 10 samples of TNF.

GOI exclusions from quality control steps were similar across all GOI panels once negative samples were removed. Remaining sample numbers with GOI relative expression data allowing quantitative analysis are tabled under "n Included" in Table 39. Despite ΔC_q being an exponential result metric, due to the molecular method is it derived from, it is commonly treated as a continuous variable for analysis purposes.

Myogenesis Genes of Interest

The ΔCq values of the myogenesis panel and co-expression correlations were examined. Lower ΔCq values represent higher expression. ΔCq values can be negative.

Table 40 - Correlation matrix displaying Pearson correlation coefficients between the myogenesis genes of interest positive expression. P-values are also displayed for each comparison.

Correlations

	Pax7	MyoD	Myf5
MyoD	0.539604		
	0.0014		
Myf5	0.099094	-0.031381	
	0.7361	0.9116	
Myog	0.799160	0.486482	-0.044873
	<0.0001	0.0048	0.8789

Cell Contents: Pearson correlation
P-Value

Table 41 - Delta cycle threshold values for the myogenesis genes of interest. ΔCq = difference between GOI in mean cycle threshold and housekeeping mean cycle threshold value. N.B. a lower ΔCq value infers a higher expression of the GOI.

Gene of Interest (GOI)	N included	ΔCq m	StDev
MyoD1	34	10.7	2.4
Pax7	46	3.0	2.1
Myf5	16	10.5	3.5
Myog	39	1.3	2.1

Results for the myogenesis GOIs showed highest expression in Pax7 and Myog, with present but lower expression of MyoD1 and Myf5 (Table 41). The samples with no detectable GOI expression were not included in this quantitative analysis.

Correlations were assessed between the myogenesis GOI expression using Pearson correlation coefficients (Table 40). Correlations were observed between Pax7 and MyoD ($r=0.539$; $p=0.001$), and with Myog relative expression levels ($r=0.799$; $p<0.001$). MyoD and Myog relative expression also correlated ($r=0.0486$; $p=0.005$). No correlations were found with Myf5 ($p>0.736$).

The cohort displayed the average physiological phenotype of a reasonably high presence of MuSCs (represented by Pax7), low division and differentiation of MuSCs into myoblasts (MyoD1 and Myf5), and a high presence of terminally differentiating, or recently created or repaired muscle fibres (Myog).

The observed relative expression correlations logically result from the presence of MuSCs and levels of MuSC activity. The lower correlation between MyoD when compared to Pax7 and Myog, suggests that the level of MuSC activation varies within the cohort. The lack of correlation between Myf5 relative expression and the other

myogenesis GOIs is likely due to the large difference in inclusion sample numbers but may relate to the shorter duration that Myf5 is expressed during MuSC turnover (25). Myf5 expression level results were only obtained for <50% of sample numbers of the other myogenesis GOIs, with melting curve anomalies from non-specific amplification accounting for all exclusions.

Senescence and Inflammatory Genes of Interest

Positively expressed senescence and inflammatory GOIs amongst the cohort showed low relative expression (Table 43) with ΔCq values above 10 cycles. Additionally, no detectable expression of CDKN2A was identified in 80% of the cohort, no IL6 in 51%, and no TNF in 20%. exclusions in the quality control steps compared to the myogenesis GOIs grouping.

Table 42 - Correlation matrix displaying Spearman rho correlation coefficients between the senescence and inflammation genes of interest. P-values are also displayed for each comparison.

Correlations

	IL6	TNF
TNF	0.547619	
	0.1600	
CDK2NA	1.000000	0.900000
	<0.0001	0.0374

Cell Contents: Spearman rho
P-Value

Table 43- Delta cycle threshold values for the senescence and inflammation genes of interest. ΔCq = difference between GOI in mean cycle threshold and housekeeping mean cycle threshold value. N.B. a lower ΔCq value infers a higher expression of the GOI. Cohort n=58 study participant samples.

Gene of Interest (GOI)	N GOI negative	N included	**ΔCq** m	StDev
CDKN2A	39	8	10.9	3.6
IL6	25	16	12.4	3.2
TNF	10	20	11.8	3.6

In the examination of panel GOI co-expression Spearman Rho rank-based test for correlation coefficients were used. Low inclusions numbers and a lack of co-expression resulted in non-parametric data. No correlation was observed between IL6 and TNF (p=0.160) (Table 42). Very strong correlations were identified between IL6, and between TNF (r=0.900) and CDK2NA (r=1.000), however the low sample numbers devalued this finding (respective overlaps for correlation calculation inclusions of n=3 and n=4 from a total of 58 patient samples).

The results indicate that the study participants had either no detectable or very low levels of senescence or inflammation within their skeletal muscle biopsies. In their alternatively expressed role as endocrine markers, they also potentially suggest systemic presence of these factors, but the reflective extent of this could not be confirmed without further samples.

Genes of interest cross-panel associations

Gene of interest cross-panel associations were examined using Spearman Rho correlation coefficients. The myogenesis GOIs relative expression levels were compared to those of the senescence and inflammation panel GOIs. Multiple strong and very strong correlations were observed between the two panels (Table 44).

Table 44 - Correlation matrix displaying Spearman rho correlation coefficients between the two genes of interest panels. P-values are also displayed for each comparison.

Correlations

	IL6	TNF	CDK2NA
Pax7	0.725403	0.609039	0.371264
	0.0022	0.0056	0.3652
MyoD	0.468275	0.352941	0.392857
	0.0783	0.1647	0.3833
Myf5	0.257143	0.806061	0.800000
	0.6228	0.0049	0.2000
Myog	0.813953	0.636739	0.626506
	0.0002	0.0045	0.0965

Cell Contents: Spearman rho
P-Value

Pax7 and Myog were found to correlate with IL6 relative expression (Pax7 – r=0.725, p=0.002; Myog – r=0.814, p<0.001). No correlation was found between IL6 and Myf5 (p=0.622). A moderate but slightly non-significant correlation was identified between IL6 and MyoD (r=0.468, p=0.078).

TNF relative expression showed positive correlations with Pax7 (r=0.609; p=0.006), Myf5 (r=0.806; p=0.005), and Myog (r=0.637; p=0.005). No correlation was found between TNF and MyoD (p=0.165). CDKN2A showed no correlation with the myogenesis GOIs (p>0.097).

In addition to its primary association with inflammation, IL6 has a parallel role as a local myokine in response to muscular physical activity (136). Increased IL6 expression in line with MuSC presence and terminal differentiation may be related to upregulation of this local function in response to certain stimuli, such as the Janus kinase / signal transducer and activator of transcription (JAK/STAT) pathway, or within certain groups (403).

Due to the association of TNF with skeletal muscle damage, the MuSC fibre repair response would be activated under these conditions. The stronger correlation with Myf5 over MyoD, while typically co-expressed at this stage in the repair cycle, may indicate preferential prolonged expression under conditions resulting in higher TNF expression. No associations were found between the myogenesis panel and the senescence marker CDK2NA.

Molecular Relative Gene Expression by Fibre Size

The relationship between the molecular relative gene expression results and the histological morphometry results were examined for associations by correlation coefficients.

The myogenesis panel of GOIs relative expression levels were compared to the immunofluorescence data results using Pearson correlation coefficients (Table 45).

Table 45 - Correlation matrix displaying Pearson correlation coefficients between the myogenesis GOI qPCR panel and sample fibre diameter and fibre ratios. As a lower ΔC_q value conveys higher relative gene expression, correlation directions should be interpreted inversely. P-values are also displayed for each comparison.

Correlations

	Pax7	MyoD	Myf5	Myog
Fib. Type Ratio	0.014996	0.081462	-0.229624	-0.230667
	0.9268	0.6631	0.4297	0.1825
Type 2 Fib. Diam.	0.025741	-0.124044	0.186554	0.226859
	0.8747	0.5062	0.5231	0.19
Type 1 Fib. Diam.	0.042651	-0.158934	-0.089967	0.20681
	0.7939	0.3931	0.7597	0.2333

Cell Contents: Pearson correlation
P-Value

No meaningful correlations were observed. Comparisons were also made to determine inflammatory and senescent correlations with muscle morphological results using Spearman rho correlation coefficients due to their results distribution (Table 46). No correlations were observed.

Table 46 - Correlation matrix displaying Spearman correlation coefficients between the inflammatory and senescent GOI qPCR panel and sample fibre diameter and fibre ratios. As a lower ΔCq value conveys higher relative gene expression, correlation directions should be interpreted inversely. P-values are also displayed for each comparison.

Correlations

	IL6	TNF	CDK2NA
Fib. Type Ratio	0.063806	-0.322949	-0.071429
	0.8284	0.1775	0.879
Type 2 Fib. Diam.	0.107811	0.125494	0.428571
	0.7137	0.6087	0.3374
Type 1 Fib. Diam.	0.149615	-0.015789	0.571429
	0.6097	0.9488	0.1802

Cell Contents: Spearman rho
P-Value

While analysing the histological sections to determine skeletal muscle fibre characteristics, an observation was made that could theoretically influence the qPCR results. Individual fibres ranged from ~30μm to ~90μm in diameter but the anatomical location of MuSCs remained constant. A larger average fibre diameter dictates a lower overall number of fibres for a finite volume of biopsy, and therefore less anatomical niche for MuSCs to reside. While the exact number of MuSCs per section was not able to be confirmed through immunofluorescence, the number per lysed sample for qPCR analysis may be influenced given standardised sizes across all samples prior to molecular analysis.

Due to the comparison of fibre diameters with myogenesis panel expression levels showing no significant associations (Table 45), this effect can be confirmed as not present and not influencing results data.

Laboratory Variables for Multivariate Analysis

All laboratory summary variables were used in further comparisons due to their concise and distinctive natures. Data was transformed where relevant to ensure data inclusion. Sample relative expression molecular data were grouped into categorical bins to facilitate the inclusion of non-detect data during multivariate modelling (404).

Chapter 6 Summary

Patient muscle anatomical immunofluorescent data showed correspondence with the accepted fibre type distribution for the quadriceps muscle group and sufficient standardisation of the biopsy methodology. This was presented for the first time in a comparably older population. Demographic correlations with fibre data were also explored, and a strong correlation was identified between fibre type diameters.

Molecular gene expression analyses showed relatively high, but varied, myogenic expression which indicated differing muscle turnover activity and propensity between study participants. This presented as high presence of MuSCs and recently created or repaired muscle fibres but low numbers of actively differentiating cells. The results also showed overall low levels of senescent and inflammatory activity within the patient muscle biopsy samples. Cross-molecular-panel investigations identified correlations between inflammation and myogenesis markers but not with senescent markers. Separately, no associations were identified between fibre anatomy and molecular gene expression profiles.

Chapter 7: Results - The Relationship between Patient Factors, Patient Physiology and Surgical Functional Outcome

Following the analysis of the individual metrics to determine their trends and associations over the course of the study, they could now be examined in a wider context.

Baseline patient reported and functional measurements were initially compared to surgical outcomes. Low pre-op PROMs scores and functional performance have previous been shown to predict poor functional outcomes post-TKA (259,260,405). Patient demographic and lifestyle factors were next compared to patient surgical outcomes, followed by an examination of the relationship between patient physiology and surgical outcomes. An investigation into the associations of patient demographic and lifestyle choices on patient physiology at time of surgery follows.

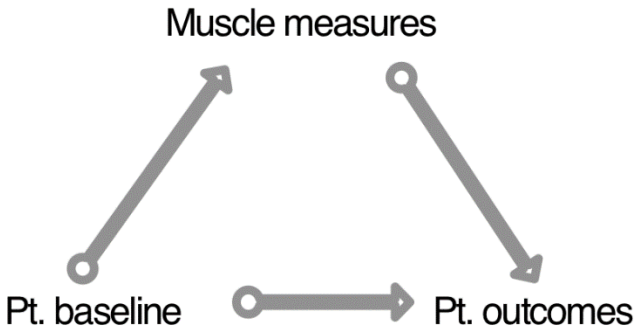

Figure 36 - Graphic overview of the multivariate modelling categories and predictive investigations. Arrow directions represent independent towards dependant variable comparisons..

The grouped variable comparisons are initially run as a combination of hypothesis driven multivariate regression models to determine contributing factors. These models are discussed in-section, with the full output displayed in Appendix C: Additional Data. For longitudinal factors, the comparisons are initially presented to evaluate the 12-month primary outcomes, followed by discussions of early recovery rates.

As complex clinical outcomes resulting from a multitude of contributors, this allowed for the many parallel and potentially compounding factors to be assessed together. With the heterogeneity of the study cohort, it also allowed for an overview of the potential contributions that each independent variable has made to a dependent primary outcome.

7.1 - Functional and Patient Reported Predictive Factors of Functional Outcome

Further Examination of Defined Baseline Time-point

The use of linear regression analysis of the individual data categories identified predictive elements amongst the study data. Variables found to be significantly different over time using paired T-tests were further analysed in this way. The overall data trend found directly measured functional metrics to be better predictors of final same-metric outcomes than patient reported outcome measurements (Table 47). Functional metrics were also generally better pre-op predictors of final function, with PROMs variables generally showing greater predictions at 6-weeks post-op of final scores. Patient step count and the EQ-5D Index PROM indicated opposing findings.

Table 47 – Comparison of intra-metric functional and PROMs factors as predictors of own final outcome score. Respective N tabled in Table 20

Predictive Factors (early result as predictor of final result at 12-months post-op)			
Factor		Initial Time-point	% of Final Result Predicted by Initial Result (Linear Regression model, R-Sq(adj), alpha = 0.05)
Functional	Leg Power (weight adj.)	Pre-op	43.7
	Leg Power (weight adj.)	6-weeks post-op	42.2
	Aggregated Locomotor Function score	Pre-op	48.8
	Aggregated Locomotor Function score	6-weeks post-op	43.4
	Step Count	Pre-op	4.3 (n.s.)
	Step Count	6-weeks post-op	20.2
PROMs	EQ-5D Index	Pre-op	17.7
	EQ-5D Index	6-weeks post-op	16.2
	Forgotten Joint Score	Pre-op	4.1 (n.s.)
	Forgotten Joint Score	6-weeks post-op	21.3
	KOOS5 Index	Pre-op	16.5
	KOOS5 Index	6-weeks post-op	27.7
	Oxford Knee Score	Pre-op	11.5
	Oxford Knee Score	6-weeks post-op	27.0

The results suggested that the best predictor of same-metric post-op outcome was pre-op Aggregated Locomotor Function (ALF) score which predicted a moderate 48.8% of final ALF score. Moderate findings were also identified from 6-week ALF scores, and from pre-op and 6-week Leg Power scores.

Despite displaying weak predictions, PROMs data did show associations which may become relevant in multivariate modelling.

Multivariate Functional and Patient Reported Predictions of Functional Surgical Outcomes

The move from individual metric analyses to multivariate models addressed elements of the complicated nature of the recovery window. Patient functional performance and PROMs scores pre-op were modelled as predictors of final (12-months post-op) and early (12-weeks post-op) outcome. The intra-metric results previously highlighted the trend whereby patients with higher scores pre-op had more favourable final outcomes. While 6-week results were also examined as predictors, this section primarily focuses on the use of pre-op markers as predictors of surgical outcomes. Pre-op scores for the functional metrics and for the PROM tools were used as potential factors influencing early and final primary metric study outcomes (Table 48).

Table 48 – Functional and PROMs variables modelled as predictors of primary metric outcomes.

Category	Variable in model
Functional	Nottingham Leg Rig Power Ratio (W/kg)
	Aggregated Locomotor Function Score (seconds)
	Average daily step count (steps)
	Op-knee range of movement
Patient Reported Outcome Measurement	Knee Injury and Osteoarthritis Outcome Score Index (KOOS5)
	EQ-5D Index
	Oxford Knee Score
	Forgotten Joint Score

General-to-specific regression modelling techniques were used (386). The multivariate model output describes contributing factors to outcome metrics (Table 49). Output significance and coefficients are displayed for contributing factors which denote contribution rank and metric influence. Model significance is also displayed along with the degrees of freedom (d.f. or ν) per model (calculated from $d.f. = n - k - 1$; n=sample number, k=number of model parameters). 'Best' models were identified as those with significant outputs with the strongest R-Sq Adj. values.

Models were identified using combined baseline functional and PROMs scores to moderately and strongly predict post-TKA outcomes at 12-weeks post-op (Table 49)

for leg power (R^2=0.73; p<0.001), ALF score (R^2=0.54; p=0.001), and step count (R^2=0.57, p<0.001). Predictive models were identified for the 12-month post-TKA leg power (R^2=0.58; p<0.001), and daily step count (R^2=0.28; p=0.005). No predictive model was identified for KOOS PROM score (p=0.495).

Table 49 - General-to-specific modelling results table for pre-op functional and patient-reported scores as predictors of early and final primary functional surgical outcomes.

Baseline (Functional and PROM) Contributing Factors to Surgical Outcomes						
Outcome Metric	Timepoint					
	12W			12M		
	Ranked contributing factors (significance; coefficient (2 s.f.))	Contribution (R-Sq Adj)	Significance (degrees of freedom)	Ranked contributing factors (significance; coefficient (2 s.f.))	Contribution (R-Sq Adj)	Significance (degrees of freedom)
Leg Power controlled by weight	Leg Power Pre-op (<0.01; +0.56) ALF Score Pre-op (<0.01; -0.02) KOOS5 Index Pre-op (0.01; -0.02) EQ-5D Index Pre-op (0.05; +0.63)	0.73	<0.001 (19)	Leg Power Pre-op (<0.01; +1.10) EQ-5D Index Pre-op (<0.01; +1.20) KOOS5 Index Pre-op (0.02; -0.022)	0.58	<0.001 (27)
ALF Timed Functional Score	ALF Score Pre-op (<0.01; +1.30) EQ-5D Index Pre-op (0.01; -31.0) KOOS5 Index Pre-op (0.01; +0.68) Daily Step Count Pre-op (0.05; 0.00)	0.54	0.001 (17)	ALF Score Pre-op (<0.01; -22.0) KOOS5 Index Pre-op (0.01; +0.47) EQ-5D Index Pre-op (0.02; -19.00) Daily Step Count Pre-op (0.05; 0.00)	0.69	<0.001 (17)
Daily Step Count	FJS Score Pre-op (<0.01; +130.0) ALF Score Pre-op (0.02; -120.0) KOOS5 Index Pre-op (0.02; -80.0)	0.57	<0.001 (19)	ALF Score Pre-op (<0.01; -170.0)	0.28	0.005 (22)
KOOS5 PROM Score	ALF Score Pre-op (<0.01; -1.20) FJS Score Pre-op (0.02; +0.59) Daily Step Count Pre-op (0.04; -0.00)	0.36	0.006 (21)	ALF Score Pre-op	-0.01	0.495 (42)

As a confirmatory measure that the correct predictive time-point metrics had been selected, 6-week PROM scores were also factored into parallel models due to their higher intra-metric prediction rates, alongside pre-op markers. All were found to be non-significant model parameters, leaving only baseline functional elements in the models. A likely factor in this finding was the higher identified intra-metric prediction rates that measured functional metrics contributed to the models compared to the patient reported factors.

For the moderate and strong prediction models created for Leg Power and ADLs, same-metric predictors featured prominently as discussed in the previous section. However, the prediction strength had greatly increased from the incorporation of baseline PROM scores. The most useful PROM tool predictors of in-clinic measurements were the EQ-5D Index and the KOOS5 Index tools which were identified as significant contributors in all models. For ALF score, baseline Daily Step Count was also found to contribute to the prediction models.

Functional and Patient Reported Predictive Factors of Functional Outcome Summary

Overall these results indicate that moderate and strong predictions of TKA post-operative early and mid-term measured functional outcome can be identified using baseline clinic measurements of leg strength and ADL function combined with a TKA specific PROM tool and a general health questionnaire. These present a simple battery of pre-operative tests that can provide a good indication of TKA functional outcome. This predictive model agrees with recent commentary on the use of PROM tools in assessing outcome and the importance of combining with functional performance based metrics to provide a full clinical picture of outcome (215).

The results correlate with previous findings by Fortin et al. that suggest that operating earlier on patients with OA, and not waiting until very late-stage disease progression and corresponding poorer function, serve to improve outcomes (255). However, the strength of the models (12-months: R^2 range 0.28-0.69) show that there is still case variation which implies that other factors are also implicated.

The models fail to identify predictive parameters of patient reported outcomes which are pertinent to determining a successful surgical outcome. The examination of wider contextualising patient background factors and physiology may elucidate closer associations.

7.2 - Patient Background Predictive Factors of Surgical Functional Outcome

Patients' clinical backgrounds and lifestyles were compared to their primary surgical outcomes following total knee arthroplasty. The variables included in these categories are tabled in Table 50 on the next page, alongside their data types and defined categorised bins. The data bins were required to allow the regression modelling to complete.

Each primary surgical outcome (Leg power, ALF score, Daily step count, and KOOS5 index PROM score) was compared as a dependent variable to the 17 independent variables, comprising patient demographic, comorbidities and lifestyle factors, using the general-to-specific modelling technique. The independent variables collectively formed the defined patient background factors.

Following the modelling of the patient primary surgical outcomes with 12-month post-op values, the technique was used to investigate contributing factors to patient very early outcomes at 12-weeks post-op. This allowed for insight to be gained into factors which correlated with favourable very early surgical outcomes. Once again this was chosen as a metric level rather than difference due to the frequently observed obstructive nature of the baseline OA pain and the uniform traumatic nature of the TKA procedure. The previous individual metric analyses discussed the changes over time for each primary metric when examined in individual patients, alongside their intra-metric associations.

Table 50 - Patient background variables modelled as predictors of primary metric outcomes. Data types and bins are stated alongside relevant comments.

Category	Subcat	Variable	Data Type	Data Bins (if appropriate)	Comment
Primary Surgical Outcomes	Primary Surgical Outcomes (12-month / 12-week value)	Nottingham Leg Rig Power Ratio (W/kg)	Continuous		Measurement of quadriceps power. Controlled by body weight.
		Aggregated Locomotor Function Score (seconds)	Continuous		Measurement of ADL function specific to lower limb surgery.
		Average daily step count (steps)	Continuous		Averaged over 4 days measured immediately following clinic visit.
		Knee Injury and Osteoarthritis Outcome Score Index (KOOS5)	Continuous		PROM score index encompassing 5 dimensions and specific to knee pathology.
Patient demographic, comorbidities, and lifestyle	Biometrics	Sex	Categorical	0 Female; 1 Male	
		Patient age at time of surgery	Interval	5 year bins e.g. 60-64, 65-69. (1, 45-49; 2, 50-54; etc.)	
		Body Mass Index at time of surgery	Interval	5 unit bins e.g. 20-24.9, 25-29.9	Kg/m^2
		Bioelectrical impedance at time of surgery	Interval	6 unit bins e.g. 20-24.9, 25-29.9	Approximation of body fat percentage
		Kellgren and Lawrence Radiographic Osteoarthritis Score	Ordinal	5 bins defined by scale (0,1,2,3,4). 0 no OA, 4 worst OA.	
		If the patient's operated knee was their dominant leg	Categorical	1 yes; 0 no	
	Comorbidities	Comorbidity - diabetes	Categorical	1 yes; 0 no	
		Comorbidity - hypertension	Categorical	1 yes; 0 no	
		History of previous arthroplasty in contralateral knee	Categorical	1 yes; 0 no	
		Long term pharmaceutical use - paracetamol	Categorical	1 yes; 0 no	
		Long term pharmaceutical use - NSAIDs	Categorical	1 yes; 0 no	
		Long term pharmaceutical use - opiates	Categorical	1 yes; 0 no	
	Lifestyle	Occupational history	Categorical	1 yes; 0 no	Divided into manual labour and sedentary occupations
		Patient alcohol group	Ordinal	3 bins based on NHS guidelines. 1, no intake; 2, intake within guidelines; 3, intake above; 4, intake more than double guidelines	Divided by NHS recommendations: zero, below 15 units/week, above 15 units/week.
		Patient smoking history	Categorical	1 a history of smoking; 0 no history of smoking.	Divided by no smoking history, and by previous or current smoking history.
		Patient peak regular activity level in life	Ordinal	10 bins based on Tegner Scale (0-10). 0 no activity possible, 10 elite sporting activity.	Categorised by modified Tegner Activity Scale
		Scottish Index of Multiple Deprivation Quintile	Ordinal	5 bins based on quintile. 1 highest deprivation, 5 lowest deprivation.	SIMD 2016 values determined by postcode

The main contributing factors, overall coefficient of multiple determination (R^2-adjusted), and statistical significance, including degrees of freedom, of the models are displayed in (Table 51), with full model outputs displayed in Appendix C: Additional Data.

Table 51 – General-to-specific modelling results table for patient background factors as predictors of early and final primary functional surgical outcomes.

Baseline (Demographic, Lifestyle, and Comorbidity) Contributing Factors to Surgical Outcomes						
Outcome Metric	**Timepoint**					
	12W			**12M**		
	Ranked contributing factors (significance; coefficient (2 s.f.))	**Contribution (R-Sq Adj)**	**Significance (degrees of freedom)**	**Ranked contributing factors (significance; Coefficient (2 s.f.))**	**Contribution (R-Sq Adj)**	**Significance (degrees of freedom)**
Leg Power controlled by weight	Peak Tegner (Physical Activity) (0.02; +0.07) Drug Hx - NSAIDs (0.13; +0.31)	0.19	0.03 (25)	Peak Tegner (Physical Activity) (0.04; +0.08) Drug Hx - Opiates (0.10; -0.36) Alcohol Hx (0.14; -0.19)	0.14	0.05 (31)
ALF Timed Functional Score	Drug Hx - NSAIDs (0.12; -11.0) Drug Hx - Paracetamol (0.16; -8.5) Drug Hx - Opiates (0.22; +7.0)	0.06	0.21 (24)	Alcohol Hx (0.02; +5.2) Manual Labour Job Hx (0.07; +6.9) SIMD Quintile (0.15; -2.1) (less deprivation showing positive effect – reduced time) Opiates (0.15; +5.5)	0.18	0.04 (30)
Daily Step Count	Age Group (0.02; -660) SIMD Quintile (0.05; +850)	0.23	0.03 (21)	Drug Hx - Paracetamol (0.01; +3000) Comorbidity - Hypertension (0.04; -2300) Smoking Hx (0.04; -2300)	0.28	0.02 (21)
KOOS5 PROM Score	Alcohol Hx (0.03; -6.4) K&L OA Score (0.03; +6.7) (worse OA pre-op) Comorbidity - Diabetes (0.11; -12.0)	0.15	0.02 (43)	K&L OA Score (<0.01; +11.0) (worse OA pre-op) Sex (0.09; -9.7) (males showing less) Peak Tegner (Physical Activity) (0.10; -1.7) Drug Hx - Opiates (0.14; -7.9) BMI Group pre-op (0.15; -3.3)	0.31	<0.001 (41)

Weak predictive models for the primary outcome metrics were identified from demographic, lifestyle and comorbid baseline factors. This included 12-week post op leg power (R^2=0.19; p=0.03), step count (R^2=0.23; p=0.03), and KOOS5 PROM score (R^2=0.15; p=0.02). No model was created for ALF at 12-weeks post-op. Viable models were identified for all 12-month primary outcomes including leg power (R^2=0.14; p=0.05), ALF score (R^2=0.18; p=0.04), step count (R^2=0.28; p=0.02), and PROM score (R^2=0.31; p<0.001).

Across all primary outcome metrics, no grouping of contributing factors was found to significantly contribute more than 31% to the predictive models. While all weak contributions, they have sufficient statistical power to allow discussion. Binary factors, by their nature, were found to contribute more than ordinal factors to each model, which is important in model interpretation. Factors are therefore ranked in respect to their significance level which reflects stronger statistical influence. For the grouping of background factors to 12-month post-op endpoint primary outcomes, all models were found to be significant. For the secondary early outcome insights provided by the 12-week measurement models, the ALF model was the only non-significant model.

During the interpretation of the models, steps were taken to avoid type 1 statistical errors resulting from classifying correlations as causations. Each contributing factor was examined in the context of the initial hypotheses and literature base, in order to qualify it as fitting current understanding, challenging current understanding, or inconclusive findings. Particular care was taken in the interpretation of factors with small influence. For each model, the significance value associated with each coefficient gave insight into its weighting in each model. A lower p-value for a contributing factor inferred a greater contribution to the multiple determination coefficient. While the alpha level for a model to be considered representative was set as 0.05, the alpha for individual factor inclusion was set to 0.20. The use of a more conservative individual factor alpha led to single viable models or multivariate models with negligible multiple determination coefficients, and to the model p-value increasing in some cases.

Patient Background as predictor of Power Output

With an overall prediction of 14%, peak physical activity, and opiate and alcohol history contributed to the prediction of primary power outcome. Peak activity was a positive contributor and alcohol and opiates did so negatively.

These findings correlate with the physiological lasting effect of training on the body following a long period of training. However, the nature of the duration of the effect requires further information as the information related to the peak regular activity

from any point in life. This may have been a few years prior or several decades. While opiates and alcohol were associated with lower normalised power output, the exact mechanism beyond associations cannot be confirmed in this study. However, the negative physiological impact of long-term use of these substances has been discussed in the literature review.

The negative coefficient within the alcohol levels of the primary model allowed for compounding as consumption increased and therefore a reduction of 0.76 in power ratio for those with the largest intake which was substantial. While at a physiological level the effects of alcohol have been shown as inhibiting, the patient group with high alcohol intake presented within the cohort with multiple factors negatively effecting outcome. For example, they reported neglecting their post-op rehabilitation recommendations in follow-up appointments, particularly those regarding improving range of movement, and were susceptible to falls and knocks to the recently replaced knee which further reduced confidence. While using the Nottingham Leg Rig instrument to measure leg power, the elements of ROM and pain guarding could account for these reductions. However, across the cohort, the vast majority were uninhibited in this regard and did not guard, which infers that the physiological effects discriminating between patient groups even within NHS alcohol intake recommendations may be strong. The model indicated that even low-level use of alcohol was associated with poorer power output outcome. Alcohol intake remained unchanged over the course of the study, which was evaluated by patient interview during clinic visits.

Long term pre-op use of opiates was detrimentally associated with primary leg power outcome. As the evidence of opiates use directly impacting on skeletal muscle physiology is inconclusive in the literature, this finding may be a surrogate for other factors, particularly at this stage in the study. Due to the existing chronic use of opiates in this patient group pre-op, there may have not been options for increasing dosage to manage pain post-op. This may have left patients with greater pain during early recovery resulting in less motivation to perform functional rehabilitation steps.

When examined during early recovery at 12-weeks post-op, peak activity was again present within the model, but this time with an influence from chronic NSAIDs use. Previous activity level played the main role in contributing 19% to the prediction of this early outcome. As discussed, the physiological elements are likely present here with the propensity to rebuild strength. These discussion points are extrapolated from an association of this specific pain relief use with outcome parameters. Alongside this, there may be psychological elements such as motivation and ability to tolerate pain. Those who had previously taken part in elite level sport were predicted to generate twice as much power (1.2W/kg) as those who had never participated in regular sporting activity (0.6W/kg).

A history of NSAIDs use was found to increase the predicted early outcome of controlled power. While physiologically NSAIDs have been shown to be potentially detrimental to MuSC activity, a positive influence was found towards power output. While a pre-op metric, this showed a large association within the early functional results which was no longer present by final outcome. This contradicted the current theoretical projections of the effect of NSAIDs on muscle turnover as discussed in the literature review. Factors such as a change in medication during this early post-op time window may have removed NSAID intake from the prescribed schedule which cannot be clarified from the available study data.

Patient Background as predictor of ADL Performance in Clinic

Contributing to a model showing 18% prediction of timed functional performance were patient alcohol history, occupational history, SIMD quintile, and a history of opiate use.

Patient alcohol intake showed the greatest impact on aggregated locomotor function score. Each grouped level of intake added 5.2 seconds to the timed performance, in a timed score with a cohort mean of 23.9 seconds. Those with a larger alcohol intake were much slower at performing the timed ADL representative activities with a likely underlying casual link.

Having an occupational history in manual labour also increased the time to complete the functional score. While such an occupational history may be equated with long term regular physical activity in terms of musculoskeletal impact history, the extent would depend on the nature of the work that was performed. As a binary metric, an occupational history of this nature adds almost 7 seconds to final ALF score outcome, which, as stated, had a mean cohort time of 23.9 seconds.

While not the strongest contributing factors, patient Scottish Index of Multiple Deprivation (SIMD) residential quintile and opiate history also affected ADL performance. A patient's performance was found to improve (reduced time taken) by 2.1 seconds for each additional quintile they belonged to. Having a history of long-term opiate use conversely increased total time by 5.5 seconds. As with previous models, the effect of long-term opiates is likely to be a surrogate for complex comorbidity and pain.

The metric of the Scottish Index of Multiple Deprivation by definition includes multiple factors includes. These include income, employment, health, education and skills, housing, crime, and geographical access to service dimensions (389). The factors are compounded and averaged with specific weighting for each geographic area to determine a national rank which is then split by quintile. Poorer performance in these domains within a patient's residential postcode was found to correlate with poorer ADL function for this outcome. In other words, higher deprivation correlated with a poorer outcome in this case.

Using the defined methodology, no viable significant model was produced to predict the very early outcome of ALF timed functional score from patient background factors ($p=0.21$).

Patient Background as predictor of Average Daily Step Count measured in the Community

Patient daily step count could be 28% predicted through a combination of pharmaceutical, comorbidity and lifestyle factors. A history of paracetamol use was found to predict increased step count, whereas hypertension and a smoking history

correlated with lower step counts. As binary factors, these predicted changed of between 2300 and 3000 steps each which had large impacts in a time-point metric average of 4600 daily steps.

For those with the physiological capability and relatively mild pain, the use of paracetamol could arguably overcome this to facilitate walking further distance. However, as a pre-op metric, this cannot be inferred at the final time-point. Despite this, the long-term use pre-operation may have enabled habitual activity of this nature. With single metric data inferring that habitual activity was a major trend in determining daily step count, having an established pre-op usual distance may have created a target for life-style continuation in a similar way.

Pharmaceutical study data from the cohort suggested that regular paracetamol use at 12-months post-op had reduced to zero in 50% of patients who responded. Extrapolated across the cohort, this infers that the habitual nature was a prominent factor, given this extended effect.

When examined to determine the factors influencing early outcome, age group and SIMD quintile were found to be relevant. Each modelled age banding reduced step count by 660 steps and each SIMD banding increased daily step count by 850 steps.

Increased age correlates with many relevant aspects that influence daily step count. Whether a core stimulating factor such as vocationally required tasks in those who still work to the physiological elements of frailty which may limit a patient's undertaking. Patients in the cohort who recorded the highest daily step counts had returned to work involving manual labour by 12 months post-op. While noticeably less impactful during weekend days, the weekday values and averaged nature of the metric resulted in an increase in these cases. However, as noted in the primary outcome model, while daily activity levels measured in this way were affected by commitments outside of the home environment, they were also affected by pre-operative habit. A long history of manageable OA impairment in the study knee or a history of multiple musculoskeletal problems may have led to a reduction in habitual activity over a longer period of time.

As with ADL performance, a correlation was observed between residential deprivation and outcome metric, but in this instance only during early recovery. A higher SIMD score theoretically correlates with easier access to services such as community health care or more generally with local amenities. Many patients in the cohort were recovering at this stage in the Scottish springtime which can be greatly affected by local council authority willingness to grit pavements amongst other steps to increase safety at this time of year, pertinent to those with mobility problems. Lower local crime rates would encourage a wider range of local activity, such as evening walking, or motivation to spend time in a town centre area. Higher educational attainment led to patients self-referring for physical therapy when they believed it was required, observed during patient clinic discussions. This was not observed elsewhere within the cohort.

Patient Background as predictor of Patient Reported Outcome (KOOS5 Index Score)

Multiple factors contributed to the model predicting 31% of KOOS5 patient reported primary study outcome at 12-months post-op. These included pre-op OA severity measured by K&L Score, patient sex, peak physical activity, history of opiate use, and pre-op BMI.

Patients reported better outcomes if they had worse radiographically evaluated OA prior to their knee replacement. For a percentage score, each level of pre-op OA severity increased the final outcome score by 11%. While testing for local physiological paracrine effect was not possible with the study investigations, established effects could in theory impact on muscle function. These were discussed in the literature review (24). However, with the diseased bone tissue removed, and 12-months elapsed since surgery, these effects would likely have dissipated. Worse OA is associated with greater arthritic pain and impairment. The contrast between extreme and relieved symptoms following arthroplasty intervention may produce a great relief than in patients with milder symptoms. This may be reflected in patient reported outcome scored in this way.

Males showed lower reported PROMs than females by 9.7% within the cohort. Study results were inconsistent with previous reports in TKA cohorts of lower PROMs scores in females (8,406,407), with this phenomenon attributed to psychosocial and psychological elements. Within the study cohort, males showed higher pre-op K&L OA scores compared to females (mean score 2.00 compared to 1.66). The higher significance of the OA scores in the model likely influences the observed sex association as a controlling factor.

Patients who had previously performed higher levels of regular physical activity marginally showed lower PROM scores. A possible explanation related to subjective evaluations of activity levels. Those who had previously experienced very high levels of activity were never going to experience these again with a prosthetic knee following TKA. For example, taking part in activities involving impact on the knee is greatly discouraged by clinical care staff as it has been shown to reduce the life of the implant (408,409). While reduced pain has improved their reported quality of life, they have not managed to reobtain access to activity levels that they previously enjoyed. This mismatch of expectations compared to reality following TKA has been shown to lead to reduced PROM scores (410–412).

Once again long-term use of opiates was found to affect outcome negatively as an indicator of comorbidities and other chronic problems. Patient reported outcomes measured by KOOS5 index were modelled as reducing by 7.9 points in these cases. Pre-op BMI group similarly negatively affected outcome, with each banding reducing reported outcome by 3.3 points. Excess patient weight continued to be associated with negative outcomes by 12-months post-op. Evaluation of BMI group at 12-months post-op to KOOS5 Index score showed that those who had subsequently lost weight (22 of 35 participants who were measured) during surgical recovery scored similarly to those who maintained similar or gained weight compared to before their operation. BMI reduction in these patients was $1.95 \pm 1.59 kg/m^2$ which may not have been sufficient to trigger the physiological benefits of greater weight loss.

Early patient reported outcome data showed similar positive correlations with OA score, as well as negative influences from alcohol and diabetes.

While not as pronounced as at 12-months post-op, pre-op OA score similarly influence PROMs during early recovery. For every alcohol use banding, patients incrementally decreased response by 6.4%, while patients with diabetes showed 12% modelled reductions with the KOOS5 Index metric. Contrastingly, in the directly measured early functional outcomes, both factors were not significant. The KOOS PROM tool utilises dimensions that cover wide areas of daily life in relation to knee function. Despite specifying this, aspects from the impact of chronic diabetes may influence patients' reports in this case. The impact from marginal alcohol use is less clear and could likely involve aspects of the physiological changes directly impacting upon the local recovery process following surgical trauma. Insight from patient biopsy physiological may elucidate underlying effects.

Patient Background as a Predictor of Surgical Outcome Summary

The multivariate models identified logical and statistically significant relationships between certain patient background factors and their subsequent early and the mid-range outcomes following TKA.

Previous peak activity level was the most determinant positive factor of power output prediction, with additional positive predictive contribution from chronic NSAIDs use. Alcohol use and opiate use were negative influences. Less residential deprivation (SIMD) was found to improve ADL performance, with alcohol use, a manual labour job history, and opiate use reducing performance. Activity in the community as assessed by daily step count was influenced by multiple factors. Age, hypertension, and smoking reduced the metric, whereas less deprivation and paracetamol were found to increase the average number of steps taken. Patient reported outcome was also affected by several factors; pre-op K&L score positively, and being male, having high previous activity levels, diabetes, being in a high BMI group, and using alcohol and opiates found to lower PROM score.

Clinic-measured direct functional outcomes (Leg power output and ALF battery of ADL performance) were noticeably more influenced by lifestyle factors with all significant modelled predictors falling into this grouping. These included pharmaceutical use, occupational and activity histories, alcohol use, and residential deprivation. The community measured functional outcome of step count and patient reported outcome were affected by a broad range of background factors also including comorbidities and biometrics alongside the lifestyle factors.

Additionally, the models only provided weak predictions (14-28%) of surgical outcomes which were much reduced in power than the previously examined functional and PROMs tool models as predictive parameters by themselves (up to 73%). Further examination of other discussed factor groupings may provide stronger models to determine the more influential factors affecting the early and very-early post-TKA surgical outcomes of leg power output, ADL performance, daily step count, and PROM score as represented by KOOS5 index.

7.3 - Patient Skeletal Muscle Physiology Predictive Factors of Functional Surgical Outcome

Patient physiological factors were compared to their primary surgical outcomes. Initially these were performed with correlation coefficients using raw data, and subsequently with general-to-specific multivariate regression modelling to ascertain contributions and potential predictive factors. The multivariate models were able to increase model sample numbers due to the creation of categorical bins for factors unrepresented by molecular ΔC_t values.

Examination of Individual Correlations between Physiology and Functional Surgical Outcomes

Due to the differences in data distributions, histological data and qPCR data for myogenic genes of interest (GOIs) were compared to the primary outcome metrics using Pearson correlation coefficients. The inflammatory and senescence GOIs were compared using Spearman rho correlation coefficients due to their lower positive sample numbers (Table 52, Table 53).

Table 52 - Correlation coefficient matric comparing early recovery primary outcome associations with physiological data.

Correlations

	KOOS5 Index (12W)	Leg Power Ratio (NLR 12W)	ALF Score (12W)	Daily Step Count (12W)
Fib. Type Ratio	-0.102936	-0.232174	0.165356	0.292782
	0.5273	0.2985	0.4621	0.2384
Type 1 Fib. Diam.	0.139919	0.381441	-0.257272	0.276282
	0.3892	0.0798	0.2477	0.2671
Type 2 Fib. Diam.	0.127469	0.329160	-0.295853	0.035894
	0.4331	0.1347	0.1813	0.8876
Pax7	-0.070288	-0.030420	-0.023168	0.134999
	0.6975	0.8959	0.9206	0.5933
MyoD	-0.307345	-0.326333	0.106935	-0.195157
	0.1350	0.2174	0.6935	0.5229
Myf5	-0.081760	-0.387049	0.087814	-0.944007
	0.8111	0.5198	0.8883	0.0560
Myog	-0.028986	0.121179	0.003023	0.185210
	0.8859	0.6212	0.9902	0.5087
Cell Contents:	*Pearson correlation*			
	P-Value			
IL6	-0.218846	0.600000	-0.400000	1.000000
	0.5436	0.4000	0.6000	<0.0001
TNF	0.078571	0.357576	-0.296970	0.035714
	0.7808	0.3104	0.4047	0.9394
CDK2NA	-0.100000	-0.500000	0.500000	-1.000000
	0.8729	0.6667	0.6667	*
Cell Contents:	*Spearman rho*			
	P-Value			

Table 53 – Correlation coefficient matric comparing endpoint primary outcome associations with physiological data.

Correlations

	KOOS5 Index (12M)	Leg Power Ratio (NLR 12M)	ALF Score (12M)	Daily Step Count (12M)
Fib. Type Ratio	-0.053878	-0.326494	0.149641	-0.076855
	0.7446	0.0899	0.4472	0.7474
Type 1 Fib. Diam.	0.149320	0.229055	-0.213525	0.311611
	0.3643	0.2410	0.2753	0.1811
Type 2 Fib. Diam.	0.190663	0.259473	-0.188349	0.176677
	0.2450	0.1824	0.3371	0.4562
Pax7	-0.209823	-0.340320	0.246826	-0.183107
	0.2412	0.0889	0.2241	0.4531
MyoD	-0.481838	-0.376097	0.225821	-0.332675
	0.0147	0.1022	0.3384	0.2257
Myf5	-0.001414	0.469627	-0.363218	-0.334418
	0.9963	0.1709	0.3022	0.5171
Myog	-0.095647	0.037858	0.174732	-0.308377
	0.6283	0.8638	0.4252	0.2285
Cell Contents:	*Pearson correlation*			
	P-Value			
IL6	-0.587879	-0.380952	0.214286	-0.100000
	0.0739	0.3518	0.6103	0.8729
TNF	0.217857	0.296703	-0.186813	0.233333
	0.4354	0.3249	0.5411	0.5457
CDK2NA	-0.657143	-0.800000	0.900000	-0.500000
	0.1562	0.1041	0.0374	0.6667
Cell Contents:	*Spearman rho*			
	P-Value			

With a set alpha of 0.05, the only observed correlation between physiological data and 12-month defined surgical outcomes was between CDK2NA and 12-month post-op ALF Score (r=0.900; p=0.037), with all others deemed non-significant. A further number of correlations were identified with alpha increased to 0.10. Weak correlations were observed between controlled maximum power output and two metrics; a larger number of type 2 fibres (r=-0.326; p=0.090) and a higher Pax7

relative expression level (r=-0.340; p=0.089). Higher explosive skeletal muscle power output, central to Nottingham Leg Rig quantified metric, is typically associated with type 2 muscle fibres and was observed in the cohort. The Pax7 results infer that a higher presence of MuSCs is correlated with higher power generation.

Separately, higher IL6 expression appeared to correlate with a higher KOOS5 index score (r=-0.588; p=0.074), but on closer inspection this may have been affected by low sample numbers (n=9). The CDK2NA results had even lower positively expressing sample numbers (n=8).

With low sample number metrics excluded (IL6 and Myf5 with daily step count), no correlations were observed at 12-weeks post-op for the majority of outcome metrics. However, a single correlation with alpha set at 0.10 was observed between type 1 fibre diameter and power output at this early stage (r=0.381; p=0.080).

The nature of the ΔC_q values presented in their raw form prevented quantitative analysis of the samples which showed no detectable relative levels of expression of the gene of interest (GOI). As such, despite viable results for the majority of participants, the number of samples for some GOIs which could be analysed in this way was reduced by as much as 84%. In reality, a lack of detectable expression was as much of a result finding as that of positive expression. For example, the lack of an expressed senescence gene marker in a patient's muscle biopsy was equal in data value to a positively expressed marker. A system to include these data for multivariate analyses was therefore established.

Regression modelling using discrete bins later allowed for a larger number of samples to be included. Allocating arbitrary values to non-detects, such as 35 or 40 cycles for C_q, when examining raw data is discouraged as it can introduce bias (404). The use of ordinal categorised bins avoided this issue as it facilitated descriptive analysis of the relative genetic expression status. The study molecular methodology utilising multiple MIQE informed quality control steps such as triplicates, removal of outliers, analysis of melting curves, and the use of multiple reference genes served to qualify the data as robust.

To gain deeper insight into the nature of the patient surgical outcomes, multivariate modelling was necessary. This allowed for increased statistical power and more representative multivariate scenarios to be tested through clear statistical steps.

Molecular expression data raw values were classified into bins for modelling. Five bins were created; non-detects, very low expression (ΔCq>10), low expression (ΔCq 5-10), medium expression (ΔCq 1-5), and high expression (ΔCq <1). The bins were allocated the numbers one to 5. As previously described, these classifications allowed for the inclusion of non-detect data thereby greatly increasing the sample numbers per GOI and the data utility. With a reference genes mean Cq of 29.9$\pm$2.9, this represented consistent and moderately high expression of mRNA. This allowed ΔCq values to be classified in this terminology. Due to the nature of the quality steps in the qPCR data processing, quality eliminations are classified the same as uniform qPCR product non-amplifications.

General-to-specific multivariate regression modelling was used as previously described to compare ten physiological baseline patient variables to their primary surgical outcomes following total knee arthroplasty. The variables included in these categories are tabled (Table 54) alongside their data types, bins, and any relevant comments. Following modelling of the primary 12-months outcomes data, this was also performed for the early outcome time-point of 12-weeks post-op. A summary of the best model findings with the ranked main contributing factors, overall coefficient of multiple determination (R^2-adjusted), and statistical significance, including degrees of freedom, are tabled in Table 55. Full model data can be found in Appendix C: Additional Data.

Table 54 - Patient physiological variables modelled as predictors of primary metric outcomes. Data types and bins are stated alongside relevant comments.

Category	Subcategory	Variable	Data Type	Data Bins (if appropriate)	Comment
Primary Surgical Outcomes (12- month / 12-week value)	Primary Surgical Outcomes (12 month / 12 week value)	Nottingham Leg Rig Power Ratio (W/kg)	Continuous		Measurement of quadriceps power. Controlled by body weight.
		Aggregated Locomotor Function Score (seconds)	Continuous		Measurement of ADL function specific to lower limb surgery.
		Average daily step count (steps)	Continuous		Averaged over 4 days measured immediately following clinic visit.
		Knee Injury and Osteoarthritis Outcome Score Index (KOOS5)	Continuous		PROM score index encompassing 5 dimensions and specific to knee pathology.
Patient baseline skeletal muscle physiology	Histological	Fibre Type Ratio	Continuous		Mean total type 1 fibres divided by mean total type 2 fibres. A higher value infers more type 1 were observed than type 2 for that patient.
		Type 1 Fibre Diameter	Continuous		um
		Type 2 Fibre Diameter	Continuous		um
	Molecular - Myogenesis	Pax7	Ordinal	5 bins based on the relative expression level of the gene (1, non-detect; 2, ΔC_t >10; 3, ΔC_t 5-10; 4, ΔC_t 1-5; 5, ΔC_t <1)	Quantified using qPCR and controlled with technical replicates and reference (housekeeping) genes
		MyoD	Ordinal	5 bins based on the relative expression level of the gene (1, non-detect; 2, ΔC_t >10; 3, ΔC_t 5-10; 4, ΔC_t 1-5; 5, ΔC_t <1)	Quantified using qPCR and controlled with technical replicates and reference (housekeeping) genes
		Myf5	Ordinal	5 bins based on the relative expression level of the gene (1, non-detect; 2, ΔC_t >10; 3, ΔC_t 5-10; 4, ΔC_t 1-5; 5, ΔC_t <1)	Quantified using qPCR and controlled with technical replicates and reference (housekeeping) genes
		Myog	Ordinal	5 bins based on the relative expression level of the gene (1, non-detect; 2, ΔC_t >10; 3, ΔC_t 5-10; 4, ΔC_t 1-5; 5, ΔC_t <1)	Quantified using qPCR and controlled with technical replicates and reference (housekeeping) genes
	Molecular - Senescence and Inflammation	CDK2NA	Ordinal	5 bins based on the relative expression level of the gene (1, non-detect; 2, ΔC_t >10; 3, ΔC_t 5-10; 4, ΔC_t 1-5; 5, ΔC_t <1)	Quantified using qPCR and controlled with technical replicates and reference (housekeeping) genes
		IL6	Ordinal	5 bins based on the relative expression level of the gene (1, non-detect; 2, ΔC_t >10; 3, ΔC_t 5-10; 4, ΔC_t 1-5; 5, ΔC_t <1)	Quantified using qPCR and controlled with technical replicates and reference (housekeeping) genes
		TNF	Ordinal	5 bins based on the relative expression level of the gene (1, non-detect; 2, ΔC_t >10; 3, ΔC_t 5-10; 4, ΔC_t 1-5; 5, ΔC_t <1)	Quantified using qPCR and controlled with technical replicates and reference (housekeeping) genes

Three viable models were identified with baseline physiological predictors of 12-month functional outcome scores. These were for leg power (R^2=0.29; p=0.01), daily step count (R^2=0.11; p=0.06), and KOOS5 PROM score (R^2=0.14; p=0.01). While not substantial predictive models (≤29%), they do indicate significantly contributing factors which merit highlighting. The modelling of the early outcomes conversely found no significant model associations or contributions of physiological factors during this stage of recovery (p≥0.13).

Table 55 - General-to-specific modelling results table for peri-operative muscle physiological factors as predictors of early and final primary functional surgical outcomes.

Baseline (Physological) Contributing Factors to Surgical Outcomes						
Outcome Metric	**Timepoint**					
	12W			**12M**		
	Ranked contributing factors (significance; coefficient (2 s.f.))	**Contribution (R-Sq Adj)**	**Significance (degrees of freedom)**	**Ranked contributing factors (significance; coefficient (2 s.f.))**	**Contribution (R-Sq Adj)**	**Significance (degrees of freedom)**
Leg Power controlled by weight	Myf5 Expression (0.24; +0.18) Fibre Type Ratio (0.31; -0.36)	0.03	0.30 (18)	MyoD Expression (<0.01; +0.47) Fibre Type Ratio (0.02; -0.90) (*Higher power with more type 2 fibres*) CDK2NA (0.09; -0.41)	0.29	0.01 (22)
ALF Timed Functional Score	Myog Expression (0.17; +2.3) TNF Expression (0.24; -4.3)	0.02	0.31 (22)	Fibre Type Ratio (0.35; +6.6) Myf5 Expression (0.45; +1.8) MyoD Expression (0.51; -2.1)	-0.07	0.72 (22)
Daily Step Count	CDK2NA (0.36; -1000)	-0.01	0.36 (19)	Myog Expression (0.06; -640)	0.11	0.06 (21)
KOOS5 PROM Score	CDK2NA (0.12; +7.0) IL6 Expression (0.20; -7.3)	0.05	0.13 (32)	Myog Expression (0.01; +4.2) TNF Expression (0.05; -7.1)	0.14	0.01 (42)

Physiological data are identified from time-of-surgery biopsies and were compared to surgical outcomes at 3 and 12 months following their arthroplasty procedure. Histological elements are considered to be relatively stable over time, with meaningful remodelling occurring in response to stimuli over periods of weeks and months, however gene expression levels can largely vary from day to day. As discussed in the literature review, selected GOIs represented gene panel trends and also cell type abundance which infer different aspects of regenerative potential or inhibition. Histological metrics and molecular genes of interest included in these models are also taken to reflect habitual undertakings as projections of future expression levels. While this cannot be confirmed without subsequent biopsy sampling to determine contemporaneous *in vivo* levels, it is a pragmatic option to gain insight in a research area with complex ethical and clinical considerations with patient interest and impact at the centre.

Baseline Muscle Physiology as predictor Power Output

Controlled leg power output at 12 months post-op was found to be 29% predicted by baseline MyoD and CDKN2A relative expression alongside quadriceps skeletal muscle fibre type proportions (Table 55).

Patient baseline MyoD expression was found to be the highest contributor to power with each subsequent expression banding adding 0.47W/kg to the body-weight-controlled score. As an intermediate gene expression within the myogenesis fibre formation and repair process, this demonstration of higher relative expression in stronger patients indicates a longer-term propensity towards myogenesis. The identification of expression perioperatively had projected effects to 12-months outcomes. However, the lack of presence when modelling the 12-week post-op values queries this finding, but that no significant model was identified for 12-week power output may allay concern (p=0.30). As stated, the use of contemporaneous biopsies is required to confirm concurrent expression.

Patients' skeletal muscle fibre composition was found to influence power output strongly, with a higher proportion of type 2 compared to type 1 skeletal muscle fibres in vastus medialis indicating a higher power output. As the instrument takes measurement of explosive maximal power output, and type 2 fibres' glycolytic fast-twitch designation, this finding was intuitive.

The relative expression of CDK2NA, an indicator of cell senescence, was found to negatively contribute to leg power output. As a proportion of cells within skeletal muscle contain this genetic expression, they are theoretically unable to function. This subsequently would impact upon factors such as muscle atrophy, leading to less recruitment, and therefore less muscle power output.

Baseline Muscle Physiology as predictor of ADL Performance in Clinic

No viable multivariate model predicting Aggregated Locomotor Function score outcomes was found representing physiological predictors of surrogate ADL performance (12-week p=0.31; 12-month p=0.72) (Table 55).

Baseline Muscle Physiology as predictor Average Daily Step Count measured in the Community

A single physiological variable was found to predict average daily step count as a 12-month post-op primary outcome (Table 55). Myogenin relative expression, was identified as predicting 11% of step count (p=0.06). Higher expression was linked to

reduced step count at 12-months post-op. Caveated with an awareness of the model's significance level (p=0.06), the finding suggests that those with recently formed or repaired myofibers at time-of-surgery are prone to lower daily step counts by 12-months post-operation. Study participants' pre-op Myogenin expression levels and pre-op step count data indicated a non-significant marginal association of lower expression correlating with higher step counts. As previously discussed, elements of habitual activity play a greater part in this outcome metric than the small projected contribution from this gene alone. Future examination of this concept could provide further insight into this effect.

Baseline Muscle Physiology as predictor Patient Background as predictor of Patient Reported Outcome

Contrastingly to the step count findings, relative Myogenin expression at baseline was found to contribute positively to patient reported outcome at primary outcome (Table 55). Additionally, TNF expression was found to contributed negatively to this outcome metric. Combined they accounted for 14% of KOOS5 PROM score at 12-months post-op.

While the role of Myogenin in the model indicated the propensity of myogenic activity having a long-term positive effect, the nature of its expression across the models warranted further investigation, with a future repeat-biopsies study design a likely candidate. The finding of TNF inflammatory marker expression at time of surgery contributing to PROM scores the following year indicated underlying long-term issues potentially related to inflammation and activity-limiting pain. The use of repeat biopsies and comparisons to time-point pain-scales in this regard may provide a clearer picture in future studies.

Summary of Physiological and Anatomical Predictive Models of Patient Surgical Outcomes

Multivariate modelling of quadriceps skeletal muscle physiology and anatomy as a predictor of TKA surgical outcomes identified a small number of significant model outputs. Leg extensor power, daily step count activity, and patient reported outcome 12-months post-op values were able to be predicted. Higher myogenin expression level positively influenced PROM score but reduced daily step count. Expression of markers of inflammation and senescence reduced PROM score and leg extensor power respectively. Positive influences on leg power were high MyoD expression and a larger proportion of type 2 'fast-twitch' muscle fibres than type 1.

The impact of background factors but lack of physiological predictors on the defined surgical outcomes (Leg Power, ADL performance (ALF), Step count, and PROMs (KOOS5)) during the early 12-weeks post-TKA recovery time-point indicates a trend which may underlie patient motivations at this stage. As background parameter predictive model findings at this time-point were weakly indicative (R^2 range 0.15-0.23) but significant ($p \leq 0.03$), psychosocial factors could play a larger role than variations in baseline physiological elements in determining patient early surgical outcome at 12-weeks post-op. The predictive strength of the background factor models suggest that, while very much relevant, other additional factors hold influence over the defined outcome metrics at this time-point. While some of these demographic, lifestyle, and comorbid factors are established in the literature base as effecting long-term outcomes, these study findings contribute to the understanding of their impact at this early 12-weeks post-TKA time-point.

With the exception of muscle power output, the same can be said for the primary 12-months post-op study outcomes. Across the range of representative outcome metrics, they are slightly more associated with the range of background factors (R^2 range 0.14-0.31) than with the measured individual physiological elements (R^2 range 0.11-0.29). As with 12-week modelling, the R^2 predictive values from these parameter groupings indicate that either combined or tertiary factors are also involved.

To impact aspects of measured functional outcomes directly, these background factors must impact on skeletal muscle physiology. The predictive modelling of identified background factors on the selected skeletal muscle physiological can provide insight into this mechanism. While the study chose a selected number of core factors, these considerations excluded a number of other relevant factors which were not investigated. Examples include diet, adherence to recommended therapy, social support, and biomechanical habits, amongst other factors which have previously been suggested as relevant to post-TKA outcome (413–415).

Insight into the multiple effects of background factors onto the physiological elements can therefore provide another broader picture of how these factors affect patients but in this instance at time of surgery, when the physiological factors were measured.

7.4 - Patient Background as a Predictor of Patient Skeletal Muscle Physiological Profile

Patient background factors including demographics, lifestyles and comorbidities were examined against quantified patient skeletal muscle physiology at time of surgery. While previously compared against the primary surgical outcomes at 12-months following TKA, the physiological measurements can be said to have elements of plasticity over this timeframe. Examination of how patient background factors can influence this perioperative physiology provides insight into what becomes a 'time-zero' for the recovery process. While the relationships of both these elements to primary outcome metrics have been examined, the insights from the direct comparisons may further elucidate the complexity of primary TKA patients' physiological phenotypes.

Variable categories were defined in previous sections and remain the same for these comparisons, with 17 background independent and 10 physiological dependent variables included. Comparisons were performed using general-to-specific multivariate regression analyses allowing for collinear and non-significant variables to be removed. Results of the best models for each physiological outcome marker are displayed in Table 56. The best models are defined as the first model to be significant below the set alpha (0.05) with the highest multiple coefficient of multiple determination.

Patient Background and Genetic Markers of Myogenesis

The multivariate modelling of patient background factors against myogenic expression produced three viable predictive models (R^2=15-21%) (Table 56). These were for Pax7, MyoD, and Myog, with no viable model for Myf5.

Patient age group featured as a positive influence in all three models. This showed that the older patients showed higher relative expression of each gene of interest. This is initially counter-intuitive as previous examination of correlation results in this study showed older individuals had smaller fibre diameters. Additionally, much previous work has shown greater propensity towards skeletal muscle atrophy and

sarcopenia in elderly patient population (416,417), particularly in regards to fibre diameter reduction and distribution, however this is not reflected in MuSC content (418).

Table 56 - General-to-specific modelling results table for patient background factors as predictors of patient peri-operative physiology.

Baseline (Demographic, Lifestyle, and Comorbidity) Contributing Factors to Patient Physiology at Time of Surgery			
Outcome Metric	**Ranked contributing factors (significance; coefficient (2 s.f.))**	**Contribution (R-Sq Adj)**	**Significance (degrees of freedom)**
Myogenesis: Pax7	Age Group (0.01; +0.21) Dominant Leg Operated (0.04; -0.72) Comorbidity - Diabetes (0.06; -1.0) Drug Hx - NSAIDs (0.09; +0.68)	0.21	0.004 (48)
Myogenesis: MyoD	Drug Hx - NSAIDs (0.05; +0.47) Alcohol Hx (0.05; +0.22) Age Group (0.07; +0.09) Arthroplasty Hx Contra Knee (0.10; -0.35)	0.15	0.017 (50)
Myogenesis: Myf5	Arthroplasty Hx Contra Knee (0.24; -0.27) Comorbidity - Hypertension (0.29; +0.26) Drug Hx - Paracetamol (0.46; +0.18) Drug Hx - NSAIDs (0.49; +0.19) Manual Labour Job History (0.77; +0.07)	-0.02	0.600 (50)
Myogenesis: Myog	Alcohol Hx (<0.01; +0.64) Age Groups (0.02; +0.25)	0.18	0.002 (53)
Senescence: CDKN2A	Age Groups (<0.01; +0.11) Arthroplasty Hx Contra Knee (0.04; -0.28)	0.24	<0.001 (54)
Inflammation: IL6	BMI Group (0.05; -0.13) Age Group (0.05; +0.07) SIMD Quintile (0.11; -0.10) Peak Physical Activity - Tegner Score (0.18; +0.04) Comorbidity - Diabetes (0.19; +0.32)	0.15	0.023 (47)
Inflammation: TNF	Arthroplasty Hx Contra Knee (0.06; -0.36) Drug Hx - NSAIDs (0.07; +0.40) Age Group (0.11; +0.07)	0.09	0.042 (54)
Histology: Fibre Type Ratio	Drug Hx - Paracetamol (<0.01; -0.31) Sex (0.03; -0.17) Dominant Leg Operated upon (0.06; 0.14) SIMD Quintile (0.11; 0.05)	0.26	0.001 (44)
Histology: Type 1 Fibre Diameter	Age Group (<0.01; -2.0) Peak Physical Activity (Tegner Scale) (0.04; -1.3) Arthroplasty Hx Contra Knee (0.04; -1.3) Drug Hx - NSAIDs (0.07; 6.237)	0.20	0.005 (45)
Histology: Type 2 Fibre Diameter	BEI Group (0.04; -2.8) SIMD Quintile (0.09; -2.5) BMI Group (0.12; +3.6)	0.11	0.046 (41)

Examination of the myogenesis process has shown that healthy elderly patients show increased expression of myogenic markers when compared to healthy middle-aged controls (419). Also previously observed in elderly individuals was the phenomenon where late myogenic marker expression is only reduced in patients with cancer cachexia and associated reduced oxidative defence (419).

Study participants' age ranged from 45 up to age 90, which represented the typical arthroplasty population, and provided a wide range for this effect to be observed. In

this cohort, data show consistent expression across the entire differentiation program in older patients, indicating an (expected) lack of cachexia and persistence of oxidative stress defence.

Other inferences from the models towards Pax7 relative expression, a surrogate for MuSC abundance, include lower expression for those whose dominated leg was operated upon, lower expression for those with diabetes, and higher expression for those who had taken long term NSAID medication.

Dominant leg surgery assumed that the leg was painful prior to surgery and likely more so than the contralateral leg. With a preferential limb inhibited by pain, the motivation for locomotor activity in general could arguably be reduced for the patient. On the other hand, with pain in a non-dominant leg, motivation for climbing a set of stairs with the dominant non-painful leg leading or other activities requiring relative limb confidence could be increased. Frequency of dominant limb TKA in the study population was 50%.

The negative influence from diabetes could be related to activity levels, aspects of vascular circulation as previously discussed regarding patient outcomes, or a general marker of poor patient global health.

The NSAID findings correlated with previous findings in studies where despite NSAID use in young athletes reducing myoblast fusion (420), they were found to improve myogenesis in the elderly (421). The ilSIRENTE study results, a large prospective cohort study of individuals in northern Italy, indicated that they have a protective effect against sarcopenia in the elderly (422). Underlying physiological mechanisms likely involve the upregulation of nuclear factor erytroid-2-related factor 2 (Nrf2), sirtuin (SIRT), and klotho factors, alongside the downregulation of the insulin-like growth factor 1 – protein kinase B – mammalian target of rapamycin 1 (IGF-1-Akt-mTOR) pathway and the focal adhesion kinase – protein kinase B – mammalian target of rapamycin 1 (FAK-Akt-mTOR) pathway (51). NSAIDs were also implicated as a significant contributing factor in relative MyoD expression, which correlated with other previously suggested model findings (122). This occurred through the inhibition

of the nuclear factor κ-light-chain enhancer of activated B cells (NFκB) transcription factor by reducing inflammatory cytokine expression (137,423,424). Both of these findings suggested that NSAIDs contribute a protective role to myogenesis and muscle health in the study TKA population.

Additional contributing factors to MyoD expression levels were alcohol history and a history of arthroplasty in the contralateral knee. Increased alcohol positively contributed to MyoD expression in contrast to expectations behind the metabolic effects of alcohol on skeletal muscle. This indicated an association between higher intake and a higher propensity to differentiate into myoblasts and undertake myogenesis activity. The negative influence of the contralateral arthroplasty was not obvious however a recent previous arthroplasty, in the order of 1-2 years prior, may have created a longer period of disuse atrophy of the patient's lower limbs than from the disuse from single leg end-stage osteoarthritis alone. The two factors may have specific individual actions as discussed or may be additional global markers of health status.

Patient Background and Genetic Markers of Senescence and Inflammation

The models for the senescence and inflammatory markers were all viable ($p \leq 0.042$) and ranged from 9% to 24% determination (Table 56). The prediction model for CDK2NA was the strongest ($R^2=0.24$), followed by IL6 ($R^2=0.15$), and then TNF ($R^2=0.09$).

Age group featured in all models as a positive influence on all the genes of interest, showing an increase of senescent and inflammatory marker with age in line with accepted correlations (425,426). Arthroplasty history in patients' contralateral knee contributed negatively to CDKN2A and TNF expression with no clear underlying suggestive principle. These individuals therefore had less senescence and inflammation as indicated by these markers.

The additional factors modelled as contributing to IL6 expression included BMI group (-ve), SIMD quintile (-ve), peak physical activity (+ve), and diabetes (+ve). As a marker of inflammation, the reduced association with weight increase contradicted current

understanding of IL6 expression increase with high fat diets (427). However, high BMI was not ubiquitously linked to high fat diets, with the effects from such diets on the autophagy pathway observed differently across muscle groups and across patient cohorts (428). Another physiological explanation could be IL6's role as a myokine (136), or alternatively with a sedentary lifestyle and a possible fatty infiltration of tissue. Similarly, the positive influence of diabetes in the model reflected its association as a stressor on the body or general health marker.

Findings showed that with less deprivation came lower IL6 expression. IL6 is connected to stresses and depression, with higher incidence in deprived areas, therefore these factors could provide the underlying influence in this element. The relationship with peak activity was unclear other than a possible tolerance to pain or habitual propensity towards active lifestyle and more frequent muscle use to the point of triggering a stress response. With these latter findings, it was important to consider the lower contribution of peak activity and diabetes to the model.

With TNF expression, the final influencer was NSAID use, with an increase in this inflammatory marker found at time of surgery following long term use of the analgesic medicine class. While pharmacological inference suggests a reduction in this cytokine, the underlying use may be in response to multiple systemic inflammatory stimuli such as other joint problems. The model contributed a predicted 9% to the relationship in this case, which leaves much room for other influencing factors in this finding.

Patient Background and Skeletal Muscle Fibre Morphometry

Patient histological factors (fibre type proportion and fibre type diameters) were modelled to background factors with significant multiple determination coefficients identified varying between 11-26% (Table 56). Variables were identified that predicted skeletal muscle fibre type ratio by 26% ($p=0.001$), type 1 fibre diameter by 20% ($p=0.005$), and type 2 diameter by 11% ($p=0.046$).

Fibre type ratio, while macroscopically a reflection of previous activity and training stimuli in an individual, were significantly influenced by four background factors.

Historical paracetamol use was associated with a bias towards more type 2 fibres, whilst being female increased the observed proportion of type 1 fibres. If the operated knee was on the patient's dominant leg then they showed a higher number of type 1 fibres, and living in a higher SIMD quintile area was also associated with a higher propensity to type 1 fibre typing.

The effect of paracetamol on skeletal muscle, as with many analgesic agents, is contested in the literature. In this cohort the association of paracetamol and type 2 fibres was an identified correlation of which the exact physiological pathways cannot be determined from this study (429,430). However, findings from recent resistance exercise studies suggest that over-the-counter dosage of paracetamol has positive effects on leg extensor power, implicating type 2 fast-twitch fibres, as measured in small groups of young male athletes (431,432). Observed physiological mechanisms implicate general reduced pain, reduced neuromuscular fatigue, and suppressed mTOR activity which leads to greater hypertrophy. The latter study observed this specifically within type 2 fibres. Confirmation of whether paracetamol use served as a general health indicative marker or whether specific pathways were implemented requires further future examination.

Female patients' propensity for a larger percentage of type 1 fibres compared to males indicated an association away from explosive glycolytic activities towards endurance oxidative stimuli. Muscle fibre types are known to differ between muscle groups in individuals and between sexes for the same muscle group. Previous findings for the quadriceps muscle grouping have also found a greater proportion of type 1 muscle fibres in females compared to males (433).

The principles underlying the associations with dominant leg and deprivation scores were unable to be determined from the data, with no clear physiological pathway. Social factors such as occupation showed no association with fibre ratio, however higher levels of recreational activity did show a non-significant propensity towards a higher type 2 fibre proportion.

Skeletal muscle fibre diameters were influenced by different variables. Type 1 fibre diameters were impacted negatively by patient age, peak activity, and previously contralateral TKA, and positively by NSAID use. Type 2 fibres were influenced negatively by BEI, and SIMD, and positively by BMI.

Notable elements of these include the NSAID association and the positive effect within the cohort, however age was a more determinant factor. For example, younger study participants with a history of long term NSAIDs use showed larger type 1 fibres compared to older participants. Having previous contralateral knee arthroplasty was not associated with patient age, and also not with NSAIDs use.

Type 2 fibres showing decreased diameter with higher body fat corroborated elements of the active lifestyle element within the study cohort data. Females are genetically predisposed to higher body fat and showed a propensity towards a higher number of type 1 fibres, which may have influenced this finding. The negative impact of less deprivation on type 2 fibre diameter correlated with the fibre ratio findings where a phenotypic preference for type 1 fibres was identified.

Summary of Patient Background Characteristics as Predictors of Baseline Muscle Physiology

Many different background factors have been implicated as contributing to physiological baseline factors. Some of these have provided influence within the grouped factor and others across multiple elements, with others showing small influence in single variables.

While all are contributors to weak models (R^2=0.09-0.26), the repeat significant appearance of multiple factors suggests causation rather than chance correlation, however this cannot be confirmed without future studies specifically targeting these sub-elements.

Patient age has appeared as an influencing factor in multiple models, as has long-term NSAIDs use. Factors such as previous arthroplasty, alcohol use, diabetes and geographic deprivation have also repeatedly influenced patient physiological factors.

Patient age was positively associated with myogenic potential alongside systemic inflammation and senescence. Preoperative long-term NSAIDs use increased expression of markers of myogenesis, but also was found to be associated with higher inflammation. Previous arthroplasty, indicating a longer history of osteoarthritis, was associated with negative myogenesis markers and negative expression in markers of inflammation and senescence. Diabetes reduced the abundance of MuSC and also reduced inflammation, but potentially also muscular activity.

The implicated physiological trends include analgesic medication marginally tipping the balance towards net growth from atrophy by inhibiting negative elements of autophagy protease and lysosome pathways and possibly preventing sarcopenia. Active lifestyles also promote myogenesis through promoted expression of Nrf2, SIRT, and Klotho, which facilitate protection against oxidative stress by reactive oxygen species. Some additional observed factors, such as the effects of alcohol and smoking, may implicate epigenetic effects. Evidence in the literature of a pathway effect is currently lacking, however observations regarding this effect may be

connected through the JAK/STAT pathway and its influence on myogenesis from local inflammatory factors such as IL6.

Once again, all models provided weak but significant predictions of response factors. The observed factors also correlate with much in the literature and provide further human evidence of some effects mostly evidenced in model studies. The weakness of the models once again hints at the complex nature of the patient population and the large number of factors influencing surgical outcome. However, the multivariate models utilising functional factors as predictors of post-TKA surgical outcome remain 'best' so far.

The explored models provided insight into the relationship between patient factors, patient physiology, and surgical outcomes however they are not exhaustive. A multitude of other factors may affect patient's functional outcome score at a certain time-point post-TKA. The models have explored the interactions and influences of significant factors that are known to impact TKA outcomes within a longitudinal study cohort. They have confirmed translated theories in a clinical cohort, identified influential hierarchies, and identified factors which have different effects in different demographic populations. Suggestions have also been made as to where further factors influencing TKA outcome may lie.

7.5 - Overarching Multivariate Prediction Models of Patient Surgical Outcome Post-TKA

The preceding sections have explored how baseline clinical and functional scores are related to post-TKA outcomes. They have considered how patient background factors and characteristics influence surgical functional outcome. The relationship between patient skeletal muscle physiology and functional outcome has been examined. And they have investigated the interactions between these two areas in how patient background characteristics are associated with patient skeletal muscle physiology and the associated influential pathway mechanisms.

Three multivariate prediction models of post-TKA functional outcome were created with varying levels of identified associations. These allowed statistical confirmation of subgroup influences and significance. All identified significant predictive factors could now be combined into an overarching predictive model which prevents the confounding issues of doing this from the beginning (overfit and loss of power). For primary 12-month post-TKA endpoint outcomes, the independent variables from each grouping which provided the most significant contributions ($p<0.05$) were utilised Table 57.

Table 57 – Patient variables modelled as predictors of primary 12-month post-TKA endpoint metric outcomes. Inclusion based upon significance contribution (<0.05) during earlier section's category-specific multivariate regression modelling.

Model	Leg Power controlled by weight	ALF Timed Functional Score	Daily Step Count	KOOS5 PROM Score
Background (Table 51)	Peak Activity (Tegner)	Alcohol Hx	Drug Hx – Paracetamol, Comorbidity - Hypertension Smoking	K&L OA Score
Physiological (Table 55)	MyoD Fibre Type Ratio	n/a	n/a	Myog Expression
Functional (Table 49)	Pre-op Leg Power Ratio, Pre-op EQ5D Index, Pre-op KOOS5	Pre-op ALF Score, Pre-op KOOS5, Pre-op EQ5D Index	ALF pre-op	n/a

General-to-specific multivariate regression modelling was used to determine the best combined predictive model for the four outcome categories (Table 58). All models created significant ($p\leq0.006$) and viable predictive outputs (R^2=0.26-0.73). Two

models with the combined variables created lower coefficients of determination than the subgroup analysis alone, one resulted in exclusions recreating a previous model, and one created a more predictive model.

Table 58 - Overarching General-to-Specific Combined Multivariate Model Results Table of Contributing Factors to Functional Surgical Outcomes. Novel models with higher R^2 values than those from previous separate sub-group models are highlighted in bold and underlined. Novel models with lower R^2 values are only underlined.

Overarching Combined Multivariate Model Contributing Factors to Functional Surgical Outcomes						
Outcome Metric	Timepoint					
	12W			12M		
	Ranked contributing factors (relationship direction; coefficient (2 s.f.))	Contribution (R-Sq Adj)	Significance (degrees of freedom)	Ranked contributing factors (relationship direction; coefficient (2 s.f.))	Contribution (R-Sq Adj)	Significance (degrees of freedom)
Leg Power controlled by weight	Leg Power Pre-op (<0.01; +0.56) ALF Score Pre-op (<0.01; -0.02) KOOS5 Index Pre-op (0.01; -0.02) EQ-5D Index Pre-op (0.05; +0.63)	0.73	<0.001 (19)	Leg Power Pre-op (<0.01; +1.10) EQ-5D Index Pre-op (<0.01; +1.20) KOOS5 Index Pre-op (0.02; -0.02)	0.58	<0.001 (27)
ALF Timed Functional Score	ALF Score Pre-op (<0.01; +1.30) EQ-5D Index Pre-op (0.01; -31.0) KOOS5 Index Pre-op (0.01; +0.68) Daily Step Count Pre-op (0.05; 0.00)	0.54	0.001 (17)	ALF Score Pre-op (<0.01; +0.80) Alcohol Hx (<0.01; +4.30)	0.60	<0.001 (30)
Daily Step Count	FJS Score Pre-op (<0.01; +100) Age Group (0.02; -530)	0.49	<0.001 (21)	Comorbidity – Hypertension (0.06; -2000) ALF Score Pre-op (0.06; -110) Smoking Hx (0.07; -2050) Drug Hx – Paracetamol (0.08; +2200)	**0.39**	0.008 (19)
KOOS5 PROM Score	ALF Score Pre-op (<0.01; -1.20) FJS Score Pre-op (0.02; +0.59) Daily Step Count Pre-op (0.04; -0.00)	0.36	0.006 (21)	K&L OA Score (<0.01; +9.89) Myog Expression (0.04; +3.18)	0.26	<0.001 (42)

The combined model to predict Leg Power at primary 12-month post-TKA endpoint eliminated all but the functional variables due to multicollinearity and statistical non-significance. This resulted in the same predictive model being created as the with the functional elements alone. This created a moderate prediction of 58% outcome with pre-op Leg Power, combined with EQ-5D and KOOS5 Index PROM scores as previously discussed.

Prediction of ALF final outcome resulted in a lower coefficient of determination but using higher degrees of freedom meaning a more powerful predictive model. The initial functional predictive model achieved a strong prediction of 69% using data

from 22 study participants. The low number out of the whole cohort was determined by the predictive use of pre-op step count as a factor which was only measured in the enhanced sub-cohort. The cross-category strongly predicted 60% of final ALF outcome using pre-op ALF score combined with patient alcohol history in 33 study participants ($p<0.001$).

Patient step count at 12-months post-op was predicted 39% with data from 25 participants using paracetamol use history, comorbidity of hypertension, smoking history, and pre-op ALF score. The model created a higher R^2 value than with the pre-op background or functional metrics alone. These both predicted 28% final outcome using 25 and 24 participants' data respectfully.

The modelling of the combined panels to predict PROM score resulted in a lower prediction (26%) than using background factors alone (31%).

Examination of combined panel predictors of early outcome at 12-weeks post-op with the same technique led to exclusions resulting in single sub-category models or with lower R^2 values than the initial single category models. Patient age combined with pre-op FJS score predicted 49% of final step count in 24 patients compared to a 57% prediction using functional factors alone in 23 patients.

These findings seemed surprising but reflected the complex and varying nature of the combined data sets, with varying numbers of responses and some missing values due to the study cohort design. For example, while all patients who attended the pre-op research clinic provided leg power data, only enhanced cohort participants had provided step count data. They also reflected the step-wise process of the general-to-specific modelling technique which utilised consistent conservative methodology.

The only combined model that improved prediction was for primary endpoint daily step count. The improved power in the model predicting final ALF score reflects the influencing effect of patient alcohol history on ADL performance, however pre-op ALF score remained the greatest influence.

Combined Summary Comments on Multivariate Model Analyses

The combined multivariate models provided further insight to the factors that influenced step count in the study cohort and identified multiple models which predicted ADL performance and KOOS5 PROM score that were not previously identified. However, the majority of these new models were not as strongly predictive as parameter sub-group models identified during earlier analysis.

Overall, the range of multivariate regression models identified in this chapter provided insight into complex clinical outcomes and their underlying contributors. Baseline functional and patient reported metrics provided the strongest prediction of early and 12-month post-op outcomes, while background and physiological factors provided weak but significant predictions of responses. They all therefore provided meaningful suggestions as to where the associated influences are found in the TKA population. Summary tables of the directional influences of factors on outcomes can be found in Appendix C: Additional Data.

Following on from the individual metrics discussion, the indications for predictive factors that influence patient 12-month post-TKA primary outcomes can be better explained when the compounding effects of multiple factors are examined. While this was not generally the case for individual physiological markers, the evidence for demographic factors and how they affect both patient baseline physiology and eventually TKA primary surgical outcome is compelling. Additionally, the identification of factors which impact upon early surgical outcome can be used to inform the clinical practice and potentially policy decisions such as regional service provision.

Considering the impact on outcome of both measured and subjective function of patients is important to generate a full picture of whether a surgical intervention has provided benefit. While on the whole predictive factors during modelling for these categories overlapped, there were instances where they diverged. For example, in the case of peak previous activity level positively influencing relative power generation but lowering patient reported outcome. The occurred due to the converging differences in positive physiological gains versus the negative mismatch

of expectations of those with higher previous sporting activity. These are important factors in equally relevant outcome measurement methodologies, but opposing impacts when using each to make an evaluation of overall TKA outcome.

Some traditional demographic factors that are well established and routinely utilised metrics showed little influence in primary patient outcomes but impacted on baseline physiology. Patient age was found to negatively associate with early step count outcome alone, despite being a positively influencing factor in the majority of baseline physiological myogenesis metrics. Similarly, patient BMI was found to only be significantly associated with patient reported function. These showed that within the cohort these traditional headline demographics are not that important in determining measured outcome, despite what they may present as in perioperative physiology.

Patient pharmaceutical history also impacted outcomes and physiological factors. Non-steroidal anti-inflammatory medication improved early leg power and also baseline physiological factors. Paracetamol positively influenced daily step count 12-months post-op. While long-term opiate use affected one 12-week post-op and one 12-month post-op outcome, it was not found to be relevant when assessing any physiological phenotypes. NSAIDs use in the TKA population overall suggests a protective effect against reduced strength and physiological markers of sarcopenia through a defence against oxidative stress.

Patient lifestyle aspects such as alcohol and tobacco use, activity levels, and deprivation levels also played substantial roles in contributing to TKA outcome scores. Alcohol use negatively impacted power output, reported outcome, and surrogate ADL performance, while smoking history negatively influenced daily step count at final time-point. Alcohol was also found to increase patient myogenic activity at baseline. Overall these two substances uphold their established positions as negative influences in health, here reflected in the arthroplasty cohort. Notable was the negative impact of any alcohol use when compared to teetotal study participants. The broad range of effects from activity levels has been commented upon, while the effects from lower deprivation levels ranged from improving reported function and

ADL performance to creating an associated baseline skeletal muscle morphological phenotype. Lower physiological stress levels in less deprived communities, higher levels of recreation, and ease of access to primary health rehabilitation and local social facilities are some of the factors identified which lead to this effect.

Clinical Implications from Modelling Outputs

To optimise patient outcome following TKA, the study results indicate that the best prediction of outcome can be made by pre-operative function, specifically leg power and surrogate ADL performance such as that assessed by the ALF test. Identifying a pathway to optimise these elements either through earlier intervention or prehabilitation would serve to increase these pre-operative values. Alcohol and tobacco use should be avoided during the first 6-months of recovery, as values plateau after this point. Consideration should be made of the prescription of NSAIDs to elderly patients to preserve muscle bulk and myogenic ability.

Activity monitoring to gain insight into step count provided an outcome metric which was significantly influenced by a number of factors during the 12-months following TKA, however was ultimately a metric that was influenced by patient habitual activity. As such, it remains an extremely useful methodology assess patient metrics in the community, with newer devices offering increased functionality with every generation, but has limited scope as a post-operative functional assessment using daily step count alone. As stated, extrapolations of this metric to include cadence and paired heart rate amongst others, would serve to bolster this method in its functional outcome use.

As a central observation, the vast majority of participants' outcomes across the cohort were positive by study endpoint at 12 months post-op. Primary TKA in response to end-stage osteoarthritis is a very established and precise surgical intervention. It has well-honed, standardised techniques and robust clinical care pathways that can respond to rare complications. However, the identified factors in these models are relevant to consider in the fine tuning of these surgical outcomes when considering the patient demographic in future practise.

Chapter 8: General Concluding Discussion and Future Work

General Concluding Discussion

This thesis followed a primary total knee arthroplasty cohort through early recovery up to 1-year following surgery while assessing surgical outcome. Performed at a single University Hospital site, with concurrent clinical and laboratory assessments by a single researcher, it evaluated the relationship between patient background and muscle physiology and the relationship with functional and patient-reported surgical outcomes.

A major underlying driver in the creation of the study was the statistic that one in five patients are dissatisfied with their surgical outcomes following primary TKA. This study targeted the early functional aspects of this finding and sought to elucidate the underpinning factors through the primary study aim:

- *"To identify preoperative and perioperative muscle factors and patient background characteristics affecting early functional outcome following primary knee arthroplasty."*

This work has identified patient background factors which were seen to influence patient post-TKA outcomes and has examined the nature of the role of patient peri-operative physiology in multifactorial determination of these outcomes.

Concluding elements are structured by their relationship to the initially set-out hypotheses and aims.

The relationship between outcome measurements

This section addresses the hypothesis that:

- *"The pattern of recovery of different categories of outcome metrics vary during the early recovery stage in primary total knee arthroplasty population."*

A successful outcome following TKA (or indeed any musculoskeletal surgical intervention) has elements of pain reduction, achievement of reasonable pre-operative expectations, and corresponding physical or functional improvement. The range of measurements performed in the cohort captured data across these parameters and provided insight into the relationship between metrics.

The range of outcome measurements covered direct functional assessments, functional assessments in the community, and patient reported outcomes. Study findings indicated that some tools evaluated outcome over the course of the study as remaining similar to pre-operative patient values, while others demonstrated large improvement when similarly assessed. For those metrics that did show improved outcome, some did so at different time-points during surgical recovery. On average, maximum functional outcome was achieved by 6-months following the procedure. Maximum measured and patient-reported ADL scores were achieved by 12-weeks post-op, and full symptoms and pain continued to improve at every time-point up until 12-months post-op. Despite tool validation during development including typical comparisons with previously established tools, the tools are not always comparable across all clinical groups and recovery time-scales.

Additionally, different motivating factors were identified in the directly measured functional outcomes. Functional outcomes measured in clinic can intrinsically have a competitive element. Whether from instructing a patient to try their hardest during a maximum power test, or by timing a battery of surrogate ADL activities. Passively measured outcomes in the community via activity monitoring potentially have an artificially motivating effect, akin to white coat syndrome (434,435), but this was less pronounced as reported by study participants given the neutral initial instructions. This difference identified the potential that while maximum leg power, ADL performance, and several PROM tools reported maximum results at 6-month post-op, patient daily step count was not a maximum possible value whenever reported. It reflected necessary or habitual activity. ADLs by definition are activities that must be performed in daily life, but modern life provides adaptions to community activity levels that can act as crutches. For example, activity-averse choices such as driving to the local shop rather than walking. The motivations for these activities can also be

affected by factors ranging from city-planning isolation to seasonal weather changes. While the 3000-5500 daily steps of the cohort's 12-month interquartile range represents substantial activity, it falls short of higher recommended targets (242). Patient and public healthy lifestyle education highlights recommended activities for continued cardiovascular health benefit (436,437), but the influence of these strategies doesn't seem to be found in the current study population. The implications for the use of step count data in clinical functional outcomes measurements was therefore highly dependent on contextual study instruction and was likely further influenced by patient baseline habitual activity level.

The difference between PROM and direct functional measurements in the first 3 months of recovery suggests differential response to patient reported function measures that don't necessarily relate to each other during this time-frame. Patients reported significantly improved function at 6-weeks following TKA which was not observed in the assessed function tests until later time-points. These findings provide important context in which early patient reported outcome data should be considered. For example, 6-weeks post-op is a well reported timeframe in clinical trials of orthopaedic devices. Studies using different tools at the same time-points cannot be reliably compared at these, despite the measurements sounding similar. These may include different functional ADL tests or different specialised PROMs tools, with all carrying some level of uncertainty when compared in this way.

The observations from this study confirm the benefit of evaluating functional outcome with multiple measurement categories (188). The use of a battery of metrics allowed for clearer evaluation of the spectrum of outcome and for the identification of aspects which were potentially causing dissatisfaction with function following primary TKA.

Core outcome sets or domains are used to standardise measurement across clinical studies and trials. OMERACT-OARSI are current working on updating a previously published set with results expected by 2021 (438,439). Defined outcome targets remain focussed on the mid-to-long term due to the contemporary longevity of prosthetics and the nature of OA as a chronic disease, but the recognition of

functional outcome variation at early time-points is still a relevant factor which should be considered by musculoskeletal researchers and developers of orthopaedic devices.

Baseline predictors of surgical outcome following primary TKA

This section addresses the hypothesis that:

- *"Patient background characteristics and muscle factors affect very early functional recovery following primary total knee arthroplasty."*

Secondary hypotheses are also addressed as stated.

A primary aim of the study was to identify factors that affect early surgical outcome post-TKA. This included various patient background factors, baseline local muscle physiology with a focus on muscle satellite cells, and other preoperative functional assessments and patient reported factors. When the baseline measurements were compared to the study's 12-weeks post-op 'early' and 12-months post-op 'final' endpoints, they allowed predictive statistical modelling to determine contribution to these surgical outcomes.

Regression modelling identified the strongest predictions of early surgical outcomes from a selection of pre-op functional and PROM scores, with weak but significant predictive factors identified amongst the physiological and background patient factors. The method also created optimal predictive models of post-operative functional and patient reported measurements by combining all available predictive variables, however these composite models offered little additional predictive accuracy beyond the initial functional and PROM score models.

- *"Patient background characteristics correlate with functional surgical outcomes following primary total knee arthroplasty."*

The strongest pre-operative predictors of patient post-operative strength (leg extensor power) utilised pre-op same-metric measured function combined with pre-

op patient reported health status (EQ-5D) and patient reported knee-specific tools (KOOS). As such, post-operative patient strength and ADL performance could be strongly predicted using pre-op values combined with EQ-5D and KOOS5 scores. This combination of metrics provides an important pre-operative measurement grouping that can be used to strongly predict 12-month outcome. Study findings also suggest that targeted rehabilitation maximising ALF-related ADL performance and leg extensor power contribute to maximum return to activity post-TKA, in conjunction with other factors.

Wider background factors were also identified that determine post-TKA surgical outcome. Previously high peak activity, chronic use of NSAIDs and paracetamol were found to associate with better directly measured outcomes, with any alcohol use, smoking, comorbidities, residential deprivation, and opiate use found to negatively affect these. Patient reported metrics were most positively affected by preperative severity of OA (assessed by K&L score) indicating a greater symptom reduction. These metrics were negatively influenced by being male, having high previous activity levels, diabetes, being in a high BMI group, and using alcohol and opiates. As such, clinic-measured direct functional outcomes were noticeably more influenced by lifestyle factors, whereas the community measured functional outcomes of step count and patient reported outcome were affected by a broad range of background factors also including comorbidities and biometrics alongside the lifestyle factors.

- *"Patient background correlates with patient muscle physiology at time of surgery in patients undergoing primary total knee arthroplasty."*

Amongst the background and baseline physiological factors, trends were observed that shed further light on the primary TKA clinical population phenotype. Long-term use of NSAIDs prior to surgery and patient age were found to have a positive influence on myogenic activity. Patients who reported a history of NSAIDs use had raised presence of MuSCs but also of inflammatory activity. With this observation, the protective effect of NSAIDs against sarcopenia was likely seen in the study population through protection against oxidative stress and possible regulation of myogenesis through the JAK/STAT pathway. Patient recreational substance use also influenced

outcome metrics with smoking history and alcohol use impacting on multiple variables. Patient residential deprivation was also found to negatively influence surgical outcomes.

Study laboratory findings identified high presence of MuSCs and recently created or repaired muscle fibres amongst patients, but low numbers of actively differentiating cells. The results also showed overall low levels of senescent and inflammatory activity within the patient muscle biopsy samples. Cross-molecular-panel investigations identified correlations between inflammation and myogenesis markers but not with senescent markers. Additionally, no associations were identified between fibre anatomy and molecular gene expression profiles.

- *"Patient muscle physiology at time of surgery correlates with functional surgical outcomes following primary total knee arthroplasty."*

Patient baseline physiological markers were only weakly predictive of post-TKA functional outcomes. Specifically, higher myogenin expression level positively influenced PROM score but reduced daily step count. Expression of markers of inflammation and senescence reduced PROM score and leg extensor power respectively. Positive influences on leg power were high MyoD expression and a larger proportion of type 2 'fast-twitch' muscle fibres than type 1.

Despite significant predictive contributions, these did not influence patient outcomes to the comparatively higher level of preoperative functional measurements or patient background factors. However, significant attribution to muscle physiological phenotype was identified from patient background factors. Additionally, the plasticity of the investigated physiological markers was identified as a likely factor in the relatively lower predictive nature of the surgical outcomes. Preoperative measurements, while significant predictors, did not provide the level of insight possible through serial contemporaneous biopsies. Future expansion of these investigations may lead to the identification of pre-operatively identifiable phenotypes that facilitate personalisation of the total knee arthroplasty procedure and recovery pathway.

The study findings provide a novel preoperative functional battery which strongly predicts early outcome following TKA. Encompassing this series of patient tests into the preoperative assessment process could help to identify patients at risk of poor outcome due to the functional factors. This stratifying step towards personalised medicine could, with the appropriate allocations of resources, continue the modern progress toward optimising recovery from total knee arthroplasty for all patients.

Future Work

There are several areas warranting future investigation. These included patient background, clinical assessment, and laboratory areas which proved linked or influential towards patient surgical outcomes. These areas can be explored in greater depth with tighter controls using different study designs or methodologies.

Patient background factors of age, comorbidities, pharmaceutical history, and deprivation were indicated as impactful on patient outcome. As a study which reflected the wider clinical population, the cohort allowed commentary on a number of relevant factors by design. Further investigations identified specific aspects of sleep, arthrogenic muscle inhibition, recreational activity levels, and ROM limitations which impacted on patient outcomes. Future studies examining all of these areas through the targeted recruitment of specific clinical cohort subpopulations can be used to elucidate the underlying effects. Generally, the robustness of study findings can always be improved through increasing study recruitment numbers whilst keeping tight controls on variables. While challenging to do both of these in a clinical cohort study, the expansion into a multisite longitudinal cohort study or performing recruitment over a longer time frame would allow for increased recruitment and also the targeting of the relevant TKA subpopulation with altered study inclusion and exclusion criteria.

While the molecular genetic expression investigated at time of surgery yielded weak correlation with final surgical outcomes, the time duration between sampling and assessment likely influenced results. A study design including longitudinal biopsies in line with post-operative assessment time-points could provide explanation as to how myogenesis, inflammatory, and senescent expression profiles correlate contemporaneously with outcome assessments. However, as demonstrated in the initial design aspirations of this study, this provides significant ethical challenges due to the infection risk and potential disabling effects that repeat biopsies can have near recent surgical sites. Care must also be taken that repeat biopsies are representative of the target muscle locations while not becoming influenced by previous scar tissue or excess variation in site.

The associations of higher alcohol use and increased myogenesis activity seemed counterintuitive and is therefore an area requiring further investigation to understand. Similarly, the significant findings of lower deprivation's association with reduced inflammation and a propensity towards an endurance muscular phenotype also warrants further study to clarify.

The expansion into the use of different laboratory methodology could also provide further information. Creating an immunofluorescent histological panel to assess myogenesis would allow for specific anatomical questions to be addressed. For example, the association of MuSCs with fibre typing, or with vasculature. Similarly, the examination of inflammatory markers or senescent expression in this way could also provide further insight into locality of expression. The potential use of flow cytometric analysis could provide in depth information on the heterogeneity of the MuSC population would clarify further hypotheses about patient physiological phenotypes. It can also be used to identify co-expression which would allow identification of cell-subpopulations. Additionally, the use of metabolomic studies, with techniques such as ultra-high-performance liquid chromatography (UPLC), mass spectrometry (MS), and nuclear magnetic resonance (NMR), could identify clearer patient physiological profiles for use in evaluating groupings, outcomes, and associations. Examples of effects to examine include drug metabolite levels, dietary metabolites, and paracrine effects resulting from arthritic disease. These would provide clearer insight into the pharmacological and dietary local effects on skeletal muscle tissue affecting control of the knee joint, and additionally further clarify the impact of arthritic severity on muscle physiology.

The study findings suggest a substantial role of muscle function in the determination of outcome following total knee arthroplasty. Insight has been delivered into the recovery process in the first year following operation, with significant differences identified between measurement tools. A battery of pre-operative assessments has been identified that strongly predicts functional outcome post-TKA. Targeted support can be provided to optimise these patient assessments.

Furthermore, patient background characteristics and analgesic use has been shown to substantially impact of this physiological patient profile. The role of specific gene expression and muscle micro-anatomy profiles in the quadriceps muscle group has next been shown to affect patient outcomes following TKA, which has set the scene for further work in this area of personalised predictions of post-TKA recovery efficacy using skeletal muscle physiology.

Appendices

Appendix A: Clinical Study Documentation

Study Methodology Development

PROMs Development

Table 59 - Total Knee Arthroplasty Prominent PROMs tools. Adapted from Ramkumar et al and Collins et al (197,198).

PROM tool description
ARS (Activity Rating Scale)
BOAS (British Orthopaedic Association Score)
Bristol Knee Score
CPG (Chronic Pain Grade)
EQ-5D (Generic Measure of Quality of Life, EuroQoL Group Index Score - 5 Domains)
Feller
FJS (Forgotten Joint Score)
HAAS (High Activity Arthroplasty Score)
HADS (Hospital Anxiety and Depression Scale)
HFKS (High-Flexion Knee Score)
Hospital for Special Surgery Knee Score
Hungerford
ICOAP (Intermittent and Constant OsteoArthritis Pain)
IKS (International Knee Society)
IPAQ (International Physical Activity Questionnaire)
KKS (Korean Knee Score)
KOOS (Knee injury and Osteoarthritis Outcome Score)
KOOS-PS (Knee injury and Osteoarthritis Outcome Score-Physical Function)
KOS-ADLS (Knee Outcome Survey - Activities of Daily Living Score)
KSS (Knee Society Score)
Lequesne
MODEMS (Musculoskeletal Outcomes Data Evaluation and Management System)
New KSS
New Zealand Score (NZS)
NHP (Nottingham Health Profile)
NRS (Numerical Rating Scale)
OKS (Oxford-12 Knee Score)
OKS-D (German adaptation)
PerF (Function) and PerP (Pain)
POMS (Profile of Mood States)
PSQ (patient satisfaction questionnaire)
SAPS (Self-Administered Patient Satisfaction)
SF-12 (Short-Form 12)
SF-36 (Short Form-36)
SF-6D (Short Form-6 Domains)
SIP (Sickness Impact Profile)
Tegner-Lysholm Knee Score
UCLA (University of California, Los Angeles)
VAS (Visual Analogue Scale)
WOMAC (Western Ontario McMaster University Score)
WORQ (Work, Osteoarthritis or joint-Replacement Questionnaire)

PROMs Tool Split by Questionnaire Battery

Study Title	Site Location	EudraCT/ Sponsor number	Principal Investigator
MAKRO	RIE / UofE	Spons: AC17048 REC: 17/SS/0088	Prof. Hamish Simpson

Included in routine and study relevant: (Data-share with consent) ✓

Study specific questionnaire (adds to routine burden): ✓

Questionnaire (PROM) timepoint	PAC		6 weeks (post-knee op.)		12 weeks		6 months		12 months	
Lothian Joint Registry / MAKRO	LJR	MAKRO	LJR	MAKRO	LJR	MAKRO	LJR	MAKRO	LJR	MAKRO
EQ-5D	✓		n/a	✓	n/a	✓	✓		✓	
OKS	✓		n/a	✓	n/a	✓	✓		✓	
FJS		✓	n/a	✓	n/a	✓	✓		✓	
KOOS		✓	n/a	✓	n/a	✓		✓		✓
COMORBIDITIES (CCI)	✓	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Patient Experience	✓		n/a	✓	n/a	✓	(very short)	✓	✓	
Patient Satisfaction		✓	n/a	✓	n/a	✓	✓		✓	
Complications			n/a	✓	n/a	✓	✓		n/a	n/a

Figure 37 - MAKRO Study Questionnaire Battery Time-Points

Clinical Study Approvals, Notices, and Documentation

NHS Health Research Authority Study Summary

The published public summary of the MAKRO Study on the NHS Health Research Authority website is as follows:

NHS
Health Research Authority

Muscular Assessed Knee Replacement Outcome (MAKRO)

Research type

Research Study

Full title

Intrinsic muscular physiological factors contributing to early outcome status after primary total knee replacement.

IRAS ID

218322

Research summary

MAKRO is a longitudinal prospective cohort study. It will research how the health of patients' upper leg muscles at time of surgery influence to their early outcomes assessments following primary knee replacement.

Quadriceps muscle biopsies from patients undergoing primary knee replacement will undergo laboratory analysis. The patients will be followed up at outpatient clinics for 12 months. Assessments will include Patient Reported Outcomes Measures and Functional Assessments to evaluate their physical performance.

The laboratory analysis will identify the health and state of the muscle cells and muscle precursor stem cells. Previous pilot work in our department has indicated that this may be a predictive factor of early outcomes.

There will also be a comparison of how lifestyle factors affect the muscle health and ability to recover.

Identification and clarification of these factors may lead to better patient management and optimisation of primary knee replacement outcomes in the future.

REC name

South East Scotland REC 01

REC reference

17/SS/0088

Date of REC Opinion

7 Aug 2017

REC opinion

Further Information Favourable Opinion

Clinical Study Research Ethics Committee Approval Document

Lothian NHS Board

South East Scotland Research Ethics Committee 01

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07 August 2017

Prof. Hamish Simpson
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Date 07 August 2017
Your Ref
Our Ref

Enquiries to: Sandra Wyllie
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Dear Prof. Simpson

Study title:	**Intrinsic muscular physiological factors contributing to early outcome status after primary total knee replacement.**
REC reference:	**17/SS/0088**
Protocol number:	**AC17048**
IRAS project ID:	**218322**

Thank you for your letter of 14 July 2017, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact hra.studyregistration@nhs.net outlining the reasons for your request.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Clinical Study Research and Development Office Approvals Document

University Hospitals Division

NHS Lothian

Queen's Medical Research Institute
47 Little France Crescent, Edinburgh, EH16 4TJ

FM/CF/approval

9 August 2017

Professor A Hamish RW Simpson
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Director: Professor Tim Walsh

Dear Professor Simpson

Lothian R&D Project No: 2017/0189 **REC No:** 17/SS/0088

Title of Research: Intrinsic muscular physiological factors contributing to early outcome status after primary total knee replacement

Participant Information Sheet:
Version 1.2, dated 31 July 2017

Consent Form:
Version 1.2, dated 31 July 2017

Protocol: Version 1.2, dated 31 July 2017

I am pleased to inform you this letter provides Site Specific approval for NHS Lothian for the above study and you may proceed with your research, subject to the conditions below.

Please note that the NHS Lothian R&D Office must be informed of any changes to the study such as amendments to the protocol, funding, recruitment, personnel or resource input required of NHS Lothian.

Substantial amendments to the protocol will require approval from the ethics committee which approved your study and the MHRA where applicable.

Please keep this office informed of the following study information:

1. Date you are ready to begin recruitment, date of the recruitment of the first participant and the monthly recruitment figures thereafter.
2. Date the final participant is recruited and the final recruitment figures.
3. Date your study / trial is completed within NHS Lothian.

I wish you every success with your study.

Yours sincerely

Fiona McArdle

Ms Fiona McArdle
Deputy R&D Director

CC: Mr Michael Pearson, General Manager, Surgical Services Directorate, RIE
Ms Caroline Whitworth, Consultant Nephrologist, Clinical Lead, Renal & Transplantation, RIE
Mr Maurice Griffin, Department of Orthopaedics and Trauma, University of Edinburgh

STA034

NHS Lothian

THE UNIVERSITY OF EDINBURGH

Participant Information Sheet

"Muscle Assessed Knee Replacement Outcome (MAKRO) study."

You are being invited to take part in a research study. Before you decide whether-or-not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Contact us if there is anything that is not clear or if you would like more information. Take time to decide whether-or-not you wish to take part.

---

1. What is the purpose of the study?

The aim is to improve our understanding of the factors that control muscle healing following injury, both from disease and its treatments.

2. Why have I been asked to take part?

You have been asked to take part as you are to undergo Open Knee Surgery in the near future.

3. Do I have to take part?

No, it is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. Deciding not to take part or withdrawing from the study will not affect the healthcare that you receive, or your legal rights.

4. What will happen if I take part?

4.1 Summary

Our research team would like to collect a small muscle tissue sample from your thigh muscle to allow us to improve the understanding of muscle healing processes.
The small samples will be analysed in a laboratory at The University of Edinburgh.

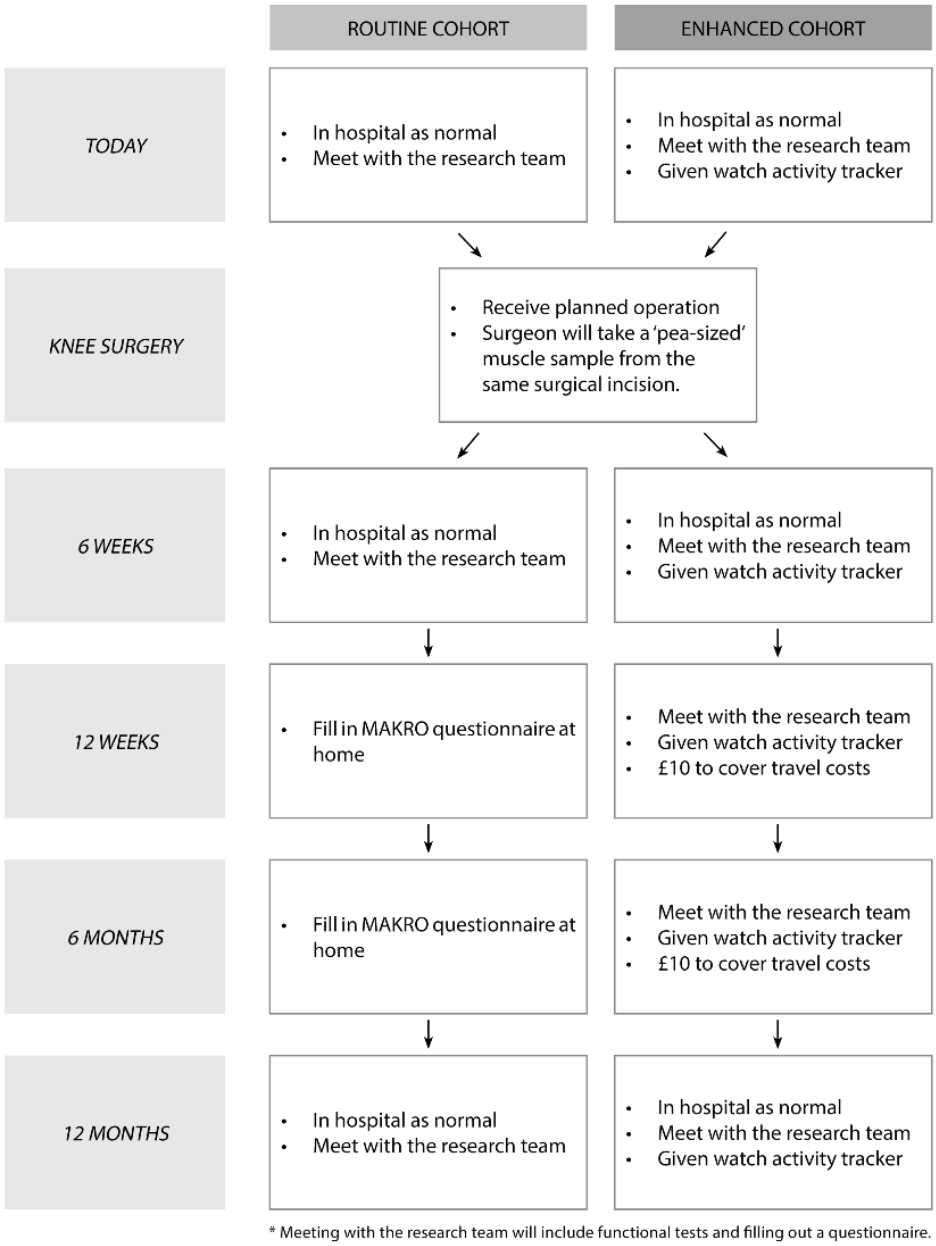
You will also be asked to fill out a questionnaire about some factors that we believe may affect the healing process. When you attend your normal follow-up clinical appointments, you will spend some of your appointment with our research staff. This may add time to your hospital visit.

You will also have the option to attend two research clinic appointments at the hospital as part of an Enhanced Cohort. This is not required for participation in the study,

The study timeline options are displayed in the flowchart on the next page.

Overview of Options and Timescales

What are my options, and what will happen when?



* Meeting with the research team will include functional tests and filling out a questionnaire.

4.2 How will this affect my surgery?
You have a hospital procedure planned which involves open surgery to your leg. During surgery, it is necessary for the surgeon to alter the soft tissues surrounding your knee. The surgeon will need to divide muscle and bone to perform the joint replacement.

We would like to have your surgeon take a 'pea-sized' sample of some of your thigh muscle that is next to your knee. This will be taken through the standard surgical opening without additional surgical procedure. This would constitute an extremely minor addition to the surgery. It will not noticeably prolong your surgery or alter your recovery. We do not believe that taking this muscle sample would inconvenience you in any way. There were zero complications in a previous research project which used the same sample procedure.

4.3 What will happen to the muscle sample?
The sample will be analysed in laboratories at the University of Edinburgh. This will help us understand how the muscle repair process is influenced.

The parts of your DNA that are relevant to muscle health will be looked at in your tissue. This information will not be relevant to your clinical team or be the kind that is used in identification. This will improve our ability to predict the recovery process. This information will be anonymised and will therefore be unable to be fed back to you as a participant.

Your small tissue sample is likely to be 'used up' through our initial project, but there is a small chance that some may be kept after the project. It may be used for future studies within our research group pending local ethical approval.

If you lose capacity (e.g. develop dementia or have a stroke), you will be withdrawn from the study. Any data or tissue already collected from you will be retained and used in the study.

Once all research is complete, the samples will eventually be discarded according to the Human Tissue (Scotland) Act 2006.

4.4 What else will I need to do?
During your normal follow up appointments at the Royal Infirmary of Edinburgh, you will also spend some time with our research staff. This will take place in the same hospital department location.

This will involve performing 10 minutes of low intensity physical functional tests. You will also be asked to provide lifestyle information, and you will fill out a questionnaire form at different time points. These will take roughly 5 minutes longer than the usual hospital questionnaires.

This will be all you need to do as part of the Routine Cohort.

Enhanced Cohort (If you choose to be part of this cohort):
As part of the Enhanced Cohort, the study will need you to make two extra visits to the hospital department in addition to your standard NHS appointments. The visits will take about 20 minutes each. They will take place at about 12 weeks- (~3 months) and 24 weeks- (~6 months) post-operation.

During these visits you will see our research staff for the same set of tests and questionnaires. We will additionally give you a watch-like activity tracker at your appointments. You will wear it for 4 days and then return it to us in a provided pre-paid envelope. You will need to keep it on your wrist all the time for 4 days. It is waterproof, and you will need to wear it while sleeping. It will provide us with a measure of step-count and other similar information.
You will be given £10 per visit to cover travel costs for these extra visits.

4.5 What happens at the end of the study?
At the end of the study you will continue to have normal clinical care from your surgical and medical team. We do not currently see any reason for the research team to contact you after the project is complete.
However, you may request to see a summary of research findings once the project is complete if you are interested. Details of this are mentioned below in Section 10.

5. What are the possible benefits of taking part?
There are no direct benefits to you taking part in this study, but the results from this study might improve the future healthcare of other patients.

You will not benefit financially from your involvement in the study. The results of this study may be used for the future commercial development of a new medicinal product, treatment or test. Your participation in this study will not entitle you to benefit financially from the company developing the product, treatment or test.

6. What are the possible disadvantages and risks of taking part?
It is not thought that there are many disadvantages to taking part in the study.

If you choose to take part in the Enhanced Cohort, you will have to attend the hospital for the additional 20 minute appointments, as mentioned above. You will be given £10 to cover travel costs for each of these extra visits.

Any incidental findings as a result of the analysis of your tissue samples, including genetic information, will not be fed back to you as the participant.

7. What if there is a problem?
If you have a concern about any aspect of this study please contact Maurice Griffin who will do his best to answer your questions.

Email (Please include "MAKRO" in subject)	Maurice.Griffin@nhslothian.scot.nhs.uk
Telephone	0131 242 6497

In the unlikely event that something goes wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against NHS Lothian but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

8. What happens when the study is finished?
At the end of the research we will dispose of used samples according to the Human Tissue (Scotland) Act 2006. Your small tissue sample is likely to be 'used up' through our initial project, but there is a small chance that some may be kept after the project. It may be used for future studies within our research group pending local ethical approval.
Data from the study will be securely kept within the department for up to ten years in line with The University of Edinburgh research policy. Any information in the University will be anonymous. Any data from the study will be published in an anonymised form

9. Will my taking part in the study be kept confidential?
All the information we collect during the course of the research will be kept confidential and there are strict laws which safeguard your privacy at every stage.

With your consent we will inform your GP that you are taking part.

To ensure that the study is being run correctly, we will ask your consent for responsible representatives from the Sponsors (University of Edinburgh and NHS Lothian) to access your medical records and data collected during the study, where it is relevant to you taking part in this research. The Sponsor is responsible for overall management of the study and providing insurance and indemnity.

Any personal identifiable information will be securely kept in NHS locations and on NHS Lothian's computer systems. Non-essential identifiers will be removed from all data while it is analysed.

10. What will happen to the results of the study?
The study will be written up as a University of Edinburgh publication and may be published in scientific journals or presented at conferences. You will not be identifiable in any published results.

We do not currently see any reason to contact you after the project is complete.
You may request to see a general summary of research findings once the project is complete. This will be available on our website:

http://www.orthopaedic.ed.ac.uk/

11. Who is organising the research and why?
This study is being sponsored by University of Edinburgh and NHS Lothian.

12. Who has reviewed the study?
The study proposal has been reviewed by ethical and academic bodies. The study has been reviewed by a University of Edinburgh Academic Committee. All research in the NHS is also looked at by an independent group of people, called a Research Ethics Committee. A favourable ethical opinion has been obtained from South East Scotland Research Ethics Committee 1 (study 17/SS/0088). NHS management approval has also been obtained.

If you have any further questions about the study please contact Maurice Griffin on: 0131 242 6497 or email: Maurice.Griffin@nhslothian.scot.nhs.uk

If you would like to discuss this study with someone independent of the study please contact:

Mr Gavin MacPherson MBChB, MFSTEd, FRCSEd (T&O)	
Email (Please include "MAKRO" in subject)	**Gavin.MacPherson@ed.ac.uk**
Telephone	**0131 242 3544**

If you wish to make a complaint about the study please contact NHS Lothian:

Patient Experience Team
2 – 4 Waterloo Place,
Edinburgh,
EH1 3EG
Tel: 0131 536 3370
feedback@nhslothian.scot.nhs.uk

Thank you for taking the time to read this information sheet.

MAKRO Study Patient Consent Form
(Enhanced Sub-Cohort Example)

NHS Lothian

CONSENT FORM

ENHANCED

"Muscle Assessed Knee Replacement Outcome (MAKRO) study."

Participant ID: ☐

Person taking consent:
Name: .....
Department: .....
Study delegation ID: .....

Please initial boxes

1. I confirm that I have read and understand the information sheet (dated 31/07/17 Version 1.2) for the above study and have had the opportunity to consider the information and ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I give permission for the research team to access my medical records for the purposes of this research study. ☐
4. I understand that relevant sections of my medical notes and data collected during the study may be looked at by approved responsible individuals from the Sponsor (University of Edinburgh and NHS Lothian), from the NHS organisation or other authorities, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
5. I agree to the retention of my anonymous data/tissue for the purpose of academic analyses including laboratory analysis, for this study. ☐
6. I agree to the retention of my anonymous data/tissue for the purpose of academic analyses including laboratory analysis, for future ethically approved studies. ☐
7. I agree to my tissue sample being used for genetic (DNA) analysis. ☐
8. I understand that my normal hospital follow up appointments must take place at the Royal Infirmary of Edinburgh. ☐
9. I acknowledge that by consenting to take part in this study, I waive any future right to benefit financially should discoveries be made of commercial value using the samples or data that I have provided. ☐
10. I agree to my General Practitioner being informed of my participation in this study. ☐

COHORT CHOICE:

I agree to take part in the above study as part of the **Enhanced Cohort.** ☐
I understand that my participation involves wearing a watch-like activity tracker and that I must return it to the hospital on each occasion after four days of use.
I understand that my participation involves attending extra hospital appointment at 12 weeks and 6 months after my knee operation and I give permission for my contact details to be given to the University of Edinburgh for this purpose.

Name of Participant (Print)	Date	Signature
Name of Person taking consent	Date	Signature

***1x** original – into Site File; **1x** **copy** – to Participant; **1x** **copy** – into medical records*

MAKRO Study Baseline Information Form

MAKRO Baseline Form

Study ID

Study op. side Left Right

Timepoint
PAC
6 weeks
12 weeks
6 months
12 months

Date of assessment

Cohort Routine Enhanced

Gender Male Female X

Forename

Date of birth

Surname

Age

Date of surgery

UHPI Number

GP Letter sent Yes No GP Letter date

CHI Number

Lifestyle

Current/previous occupation

Sedent Labour

Other lower body arthroplasty Y N Contra Knee Contra Hip Ipsi Hip Contra Ank Ipsi Ank Other

Xray score

Dominant side (L/R)

Smoking (cigs per day)

Alcohol (units per week)

Bloods FBC-Haemoglobin (g/L) U&E - Urea (g/L) Potassium (mmol/L) Creatinine (µmol/L)

LFTs - AlkPhos (U/L) GammaGT (U/L) ALT (U/L) Billirubin (µmol/L)

CCI comments
from routine PAC qb

European Tegner

Peak fitness

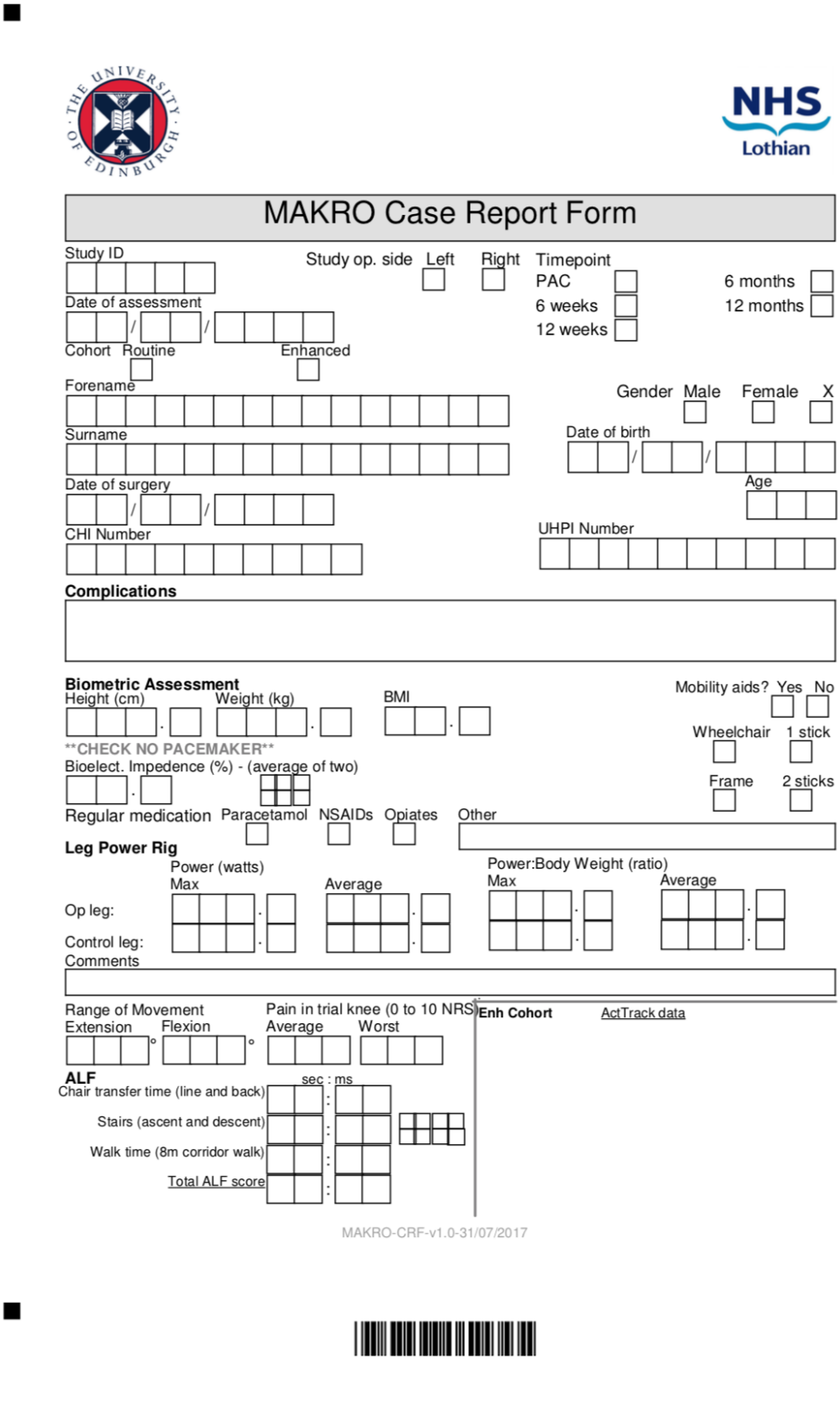
Last year

Currently

Comments

MAKRO-BF-v1.2-31/07/2017

MAKRO Study Case Report Form



MAKRO Case Report Form

Study ID

Study op. side Left Right

Timepoint
PAC
6 weeks
12 weeks
6 months
12 months

Date of assessment / /

Cohort Routine Enhanced

Forename

Gender Male Female X

Surname

Date of birth / /

Date of surgery / /

Age

CHI Number

UHPI Number

Complications

Biometric Assessment

Height (cm) . Weight (kg) . BMI .

Mobility aids? Yes No

Wheelchair 1 stick

Frame 2 sticks

CHECK NO PACEMAKER

Bioelect. Impedence (%) - (average of two) .

Regular medication Paracetamol NSAIDs Opiates Other

Leg Power Rig

	Power (watts) Max	Power (watts) Average	Power:Body Weight (ratio) Max	Power:Body Weight (ratio) Average
Op leg:	.	.	.	.
Control leg:	.	.	.	.

Comments

Range of Movement
Extension ° Flexion °

Pain in trial knee (0 to 10 NRS)
Average Worst

Enh Cohort ActTrack data

ALF

	sec : ms
Chair transfer time (line and back)	:
Stairs (ascent and descent)	:
Walk time (8m corridor walk)	:
Total ALF score	:

MAKRO-CRF-v1.0-31/07/2017

European (Scottish) Tegner Activity Score

Please indicate the HIGHEST level of activity that you have participated in:

- AT PEAK FITNESS WHEN YOU WERE YOUNGER
- LAST YEAR BEFORE YOUR SURGERY
- CURRENTLY

LEVEL	ACTIVITY
Level 10	**Competitive sports:** Football, rugby, acrobatics (national elite).
Level 9	**Competitive sports:** Football, rugby (lower divisions), hockey, wrestling, gymnastics, basketball, figure-skating.
Level 8	**Competitive sports:** Squash or badminton, track and field athletics (jumping, etc.), down-hill skiing, taekwondo. **Recreational sports:** Mogul skiing.
Level 7	**Competitive sports:** Tennis, running, motorsport, handball, volleyball. **Recreational sports**: Football, rugby, hockey, basketball, squash, running.
Level 6	**Competitive sports:** Snowboarding. **Recreational sports:** Tennis and badminton, handball, volleyball, down-hill skiing, jogging at least 5 times per week.
Level 5	**Competitive sports:** Cycling, cross-country skiing, fencing. **Recreational sports:** Jogging on uneven ground at least twice weekly, snowboard, telemark skiing. **Work:** Heavy labor (construction, fireman etc.)
Level 4	**Recreational sports:** Cycling, cross-country skiing, jogging on even ground at least twice weekly, boxing, weight lifting, aerobics. **Work:** Moderately heavy labor (e.g. truck driving, etc.)
Level 3	**Competitive/recreational sports:** Swimming, walking in forest possible, dancing, table tennis, windsurfing. **Work:** Light labor (nursing, etc.)
Level 2	**Recreational sports:** Walking on uneven ground, golf, bowling, curling, sailing, horse riding. **Work:** Light labor (e.g. shop assistant, school teacher).
Level 1	**Recreational sports:** Walking on even ground, bridge, archery, canoeing, shooting. **Work:** Sedentary (barber, office work etc.)
Level 0	**Sick leave or disability pension because of knee problems.**

Appendix B: Laboratory Protocols

Histology

Immunohistochemistry Overview

Specific biological tissues can be identified through histological stains and counterstains, but this can become complicated when specificity is required. Immunohistochemistry allows for the visualisation of specific antigens on target tissues. It is superior to histology in that it can identify different targets with different stains and can do so with much greater precision. It can identify colocalization of different antigens, for example in order to identify a sub-phenotype within a cell population. Primary antibodies raised against a target protein are incubated on a slide section, followed by an incubation period of a counterstain or a secondary antibody against the primary.

Immunohistochemistry relies upon antigen-antibody interactions to identify certain structures or presence of certain protein expression. The antibody variable domain is the geometric and chemical complement of the antigen epitope which allows for highly targeted specificity (440).

While immunohistochemistry is very effective for some uses, it is limited due to its restriction to light microscopy. When trying to visualise colocalization of multiple targets, a colour blend at a specific location can be difficult to identify when present in differing concentrations. For example, both targets may be present, but one may exist in higher abundance and obscure the other. While altering antibody concentrations can help with balance, it can never be definitive. A variation of the immunohistochemical technique can provide this through adaption of the secondary antibody.

Immunofluorescence Overview

Immunofluorescence (IF) staining allows for the tagging of antigen targets with secondary fluorescent probes. Choosing probes with emission spectra at differing wavelengths allows for visualisation of each targets separately with fluorescence microscopy. Each desired target in the tissue has been labelled with different fluorophore, they can be separately imaged using a fluorescence microscope. The images can then be digitally combined into merged images that allow for accurate identification of colocalization of targets. Similarly, the technique can be used to identify anatomical landmarks which may help with identifying or categorizing structures.

Haematoxylin and Eosin materials and staining protocol

Haematoxylin and Eosin (H&E) Histology Stain for human muscle

Perform in hood due to alcohols. Use gloves and lab coat.

Summary

Histological stain to identify general structure of the sample: fibre size and contours, position of nuclei, fibrosis, inflammation, nerves, blood vessels.
For snap frozen or fixed samples in paraffin.

Read all MSDS, Risk Assessment, and be aware of PPE and mechanical controls for this protocol before beginning.

Equipment	Instruments		Materials	
• 11 histology staining dishes • Rack for slides to fit in dishes • Sink with running cold tap water • Timer	Disposable forceps Paper towel roll Coverslips appropriate to size of sample.	X1 X1 X*n*	• VFM Harris Haematoxylin Stain (Acidified) – RBA-4202-00A. • Eosin Y Stain (Aqueous) – RBC-0100-00A. • Ethanol absolute – 20821.330 • Histochoice clearing agent – H2779. • DPX mountant – O6522.	1L 1L 2.5L 1L 100/500mL

Solution(s)		Method
Haematoxylin Stock	 200mL	Pour 200ml from stock bottle.
Eosin Stock	 200mL	Pour 200ml from stock bottle.
Histochoice Stock	 400mL	Pour 200ml from stock bottle into each (2) staining dish.
Alcohol gradient Ethanol dH_2O	 990mL 210mL	50% - 100ml EtOH, 100ml dH_2O 70% - 140ml EtOH, 60ml dH_2O 80% - 160ml EtOH, 40ml dH_2O

		95% - 190ml EtOH, 10ml dH_2O 100% - 200ml EtOH 100% - 200ml EtOH

***NOTE:* Use distilled Milli-Q water or equivalent in all protocol steps and for preparing all solutions.**
***NOTE:* All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly. Surgical equipment may be sterilized by simply soaking in 70% ethanol; however, it is important to rinse in PBS before use since ethanol will "fix" the tissue.**

For those new to Histological techniques:

a. Familiarise yourself with the work environment (e.g. get comfortable and practice technique).
b. Be mindful of hood or biosafety cabinet function/use; airflow disturbance, contaminants, working sliding sash height, and substances that may compromise the HEPA filters. Be aware of the location of all instruments and materials within the cabinet.
c. Familiarise yourself with the biochemistry theory behind the stains, and what correct staining should look like.

Methods

A. Can be perfomed on fixed or snap frozen tissue. *(Separate in-depth guides are available for the following)*

- *FIXED: 4% Paraformaldehyde (PFA) for 24 hrs then 70% EtOH until embedded in paraffin. Store at room temperature.*
- *SNAP FROZEN: Isopentane (2-Methylbutane) cooled in liquid nitrogen (cool isopentane until solid – remove onto bench until partial liquid then snap freeze samples.) Store in -80°C freezer. Thaw sample once (repeat cycles = artefact).*

B. Prepare solutions. (See 'Solutions' section for more detail).

- *Harris Haematoxylin Stain* *(100% from stock)*
- *Eosin Y Stain (Aqueous)* *(100% from stock)*
- *Ethanol dilutions* *(make up 200ml for Histo StainDish)*
- *Histochoice clearing agent* *(100% from stock)*
- *Keep one Histological Staining Dish in sink for running tap water rinses.*

C. Sectioning.

- *Cut tissue as preferred – microtome (fixed and paraffine embedded) or cryostat (snap frozen – be careful with thawing).*
- *Superfrost Plus slides preferred to prevent sections lifting during staining protocol.*
- *Label slides with standard format in pencil: Date, Name, Experiment, Sample number etc.*

Staining.

- *Rack up slides to be stained.*
- *Fixed in paraffin:*
  i. *Dewax paraffin sections – incubate slides at 60°C for 20 minutes.*
  ii. *Dewax – Histochoice wash for 3 minutes (repeat X2).*
  iii. *Rehydrate sections – 100% ethanol for 3 minutes (repeat X2).*
  iv. *Rehydrate sections – 70% ethanol for 3 minutes.*

  Continue as below…

  Perform all following steps in a hood (bar sink washes)

- *Snap frozen:*
  i. *Stain – Haematoxylin for 30 seconds.*
  ii. *Remove excess stain in running tap water ~2 mins.*
  iii. *Stain – Eosin for 30 seconds.*
  iv. *Remove excess stain in running tap water ~2 mins.*
  v. *Dehydrate – 50% EtOH – 30 seconds.*
  vi. *Dehydrate – 70% EtOH – 30 seconds.*
  vii. *Dehydrate – 80% EtOH – 30 seconds.*
  viii. *Dehydrate – 95% EtOH – 30 seconds.*
  ix. *Dehydrate – 100% EtOH – 30 seconds.*
  x. *Dehydrate – 100% EtOH – 30 seconds.*
  xi. *Clear – Histochoice – 30 seconds.*
  xii. *Clear – Histochoice – 30 seconds.*
- *Mount with coverslip:*
  i. *Remove slides from rack and allowed to evaporate briefly on paper towel.*
  ii. *Add small drop of DPX mountant on to section.*
  iii. *Apply coverslip.*
  iv. *Leave to dry in hood before examining under microscopy.*

END

ATPase Histology Stain for human muscle fibre types

Perform in hood due to odour and vapour. Use gloves and lab coat. Keep hood light off to protect from light.

Summary

Histological stain to identify different fibre types in human muscle tissue. Type 2 fibres will become dark, and type 1 fibres will be lighter.

Read all MSDS, Risk Assessment, and be aware of PPE and mechanical controls for this protocol before beginning. Fresh Snap Frozen muscle samples only. Will not work on fixed samples as is metabolic.

Equipment	Instruments		Materials	
• 11 histology staining dishes • Rack for slides to fit in dishes • Sink with running cold tap water • Timer • Dry incubator set to 37°C.	Disposable forceps Paper towel roll Coverslips appropriate to size of sample.	X1 X1 X*n*	• Ethanol absolute – 20821.330 • Histochoice clearing agent – H2779. • DPX mountant – O6522. • Glycine – G8898 • NaCl – S7653 • ATP – A2383 • NaOH - 221465 • Ammonium Suphide - A1952 • Cobalt Chloride – C8661	2.5L 1L 100/500mL

Solution(s)		**Method**
Incubating Solution (ATP)		Dissolve glycine, NaCl, and $CaCl_2$ in dH_2O. Use pH meter. Adjust pH to 9.4 using gradual addition of 0.1M NaOH. Once at 9.4, add ATP. *Leave incubating solution in 37°C incubator to acclimatise.*
Glycine	0.915g	
NaCl	0.714g	
$CaCl_2$	2.64g	
dH_2O	146ml	
0.1M NaOH	~50-65ml (see method)	
ATP	0.100g	
Cobalt Chloride (2% solution) (w/v)	*200ml*	Dissolve $CoCl_2$ in dH_2O with magnetic stirrer.
Cobalt Chloride	4g	
dH_2O	200ml	
Ammonium Sulphide (1%)		Combine liquids. Perform in fume hood as strong odour.
Ammonium Sulphide	2ml	
dH_2O	198ml	
0.1M NaOH	*1L*	Dissolve pellets in dH_2O.
NaOH pellets	4g	
dH_2O	1L	
Histochoice		Pour 200ml from stock bottle into each (2) staining dish.
Stock	400mL	
Alcohol gradient		50% - 100ml EtOH, 100ml dH_2O 70% - 140ml EtOH, 60ml dH_2O 80% - 160ml EtOH, 40ml dH_2O 95% - 190ml EtOH, 10ml dH_2O 100% - 200ml EtOH 100% - 200ml EtOH
Ethanol	990mL	
dH_2O	210mL	

NOTE: **Use distilled Milli-Q water or equivalent in all protocol steps and for preparing all solutions.**

NOTE: **All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly. Surgical equipment may be sterilized by simply soaking in 70% ethanol; however, it is important to rinse in PBS before use since ethanol will "fix" the tissue.**

For those new to Histological techniques:

a. Familiarise yourself with the work environment (e.g. get comfortable and practice technique).
b. Be mindful of hood or biosafety cabinet function/use; airflow disturbance, contaminants, working sliding sash height, and substances that may compromise the HEPA filters. Be aware of the location of all instruments and materials within the cabinet.
c. Familiarise yourself with the biochemistry theory behind the stains, and what correct staining should look like.

Methods

A. Must be performed on fresh, snap frozen muscle sample. Not on fixed tissue.

- *Collect tissue in advance..*
- *SNAP FROZEN: Isopentane (2-Methylbutane) cooled in liquid nitrogen (cool isopentane until solid – remove onto bench until partial liquid then snap freeze samples.) Store in -80°C freezer. Thaw sample once (repeat cycles = artefact).*

B. Prepare solutions.

- *See 'Solutions' section for more detail.*
- *Ammonium sulphide [$(NH_4)_2S$] and Cobalt Chloride [$CoCl_2$] can be made in advance.*
- *Incubating solutions must be made up fresh.*
- *If $(NH_4)_2S$ is yellow then needs changed. Similarly with excess surface precipitate with $CoCl_2$.*
- *Keep one Histological Staining Dish in sink for running tap water rinses.*

C. Sectioning.

- *Cut tissue – cryostat (snap frozen – be careful with thawing).*
- *Superfrost Plus slides preferred to prevent sections lifting during staining protocol.*
- *Label slides with standard format in pencil: Date, Name, Experiment, Sample number etc.*

Staining.

- *Rack up slides to be stained.*

  Perform all relevant following steps in a hood.

- *Snap frozen:*
  i. *Incubating solution for 30 minutes at 37°C.*
  ii. *Remove excess stain in dH_2O bath ~2 mins.*
  iii. *Stain – 2% Cobalt Chloride for 5 mins.*
  iv. *Remove excess stain in running tap water ~1 min.*
  v. *dH_2O bath for ~1 min.*
  vi. *dH_2O bath for ~1 min.*
  vii. *Stain – 1% Ammonium Sulphide for 1 min. (***be aware of odour *limitation)***
  viii. *Remove excess stain in running tap water ~4 mins. (***be aware of odour *limitation)***
  ix. *Dehydrate – 50% EtOH – 30 seconds.*
  x. *Dehydrate – 70% EtOH – 30 seconds.*
  xi. *Dehydrate – 80% EtOH – 30 seconds.*
  xii. *Dehydrate – 95% EtOH – 30 seconds.*
  xiii. *Dehydrate – 100% EtOH – 30 seconds.*
  xiv. *Dehydrate – 100% EtOH – 30 seconds.*
  xv. *Clear – Histochoice – 30 seconds.*
  xvi. *Clear – Histochoice – 30 seconds.*
- *Mount with coverslip:*
  i. *Remove slides from rack and allowed to evaporate briefly on paper towel.*
  ii. *Add small drop of DPX mountant on to section.*
  iii. *Apply coverslip.*
  iv. *Leave to dry in hood before examining under microscopy.*

END

Pax7 Immunofluorescence Stain for human muscle satellite cells

Summary

Immunofluorescence stain to identify the Pax7 nuclear marker representing muscle satellite cells in human muscle tissue. Secondary fluorophore labelling provides identification in the TRITC channel.

Equipment	Instruments		Materials
• 800W microwave • Pressure cooker (Nordic Ware Tender Cooker) • Slide staining rack • Histology staining dishes • Rack for slides to fit in dish • Sink with running cold tap water • Timer • Dry incubator set to 37°C.	Disposable forceps Paper towel roll Coverslips appropriate to size of sample.	X1 X1 X*n*	• Pax7 (PAX7-s) primary antibody (DHSB) • Donkey anti-mouse TRITC (PA128625) (Fisher Scientific) • Vectashield with DAPI (H-1200) (Vector Labs) • Antigen unmasking solution (H-3300) (Vector Labs)

Solution(s)		**Method**
Antigen Retrieval Buffer		Add unmasking solution to PBS and mix with magnetic stirrer.
Antigen unmasking solution (H-3300)	15ml	
Phosphate Buffered Saline X1	150ml	
0.05% PBST		Add 500µl of Tween 20 to 1L of X1 PBS.
Phosphate Buffered Saline X1	1000ml	
Tween 20	500µl	

Methods

A. Prepare solutions.
- *See 'Solutions' section for more detail.*
- *Antigen retrieval buffer can be made in advance.*

B. Sectioning.
- *Cut tissue – cryostat (snap frozen – be careful with thawing).*
- *Superfrost Plus slides preferred to prevent sections lifting during staining protocol.*
- *Label slides with standard format in pencil: Date, Name, Experiment, Sample number etc.*

Staining.
- *Rack up slides to be stained.*
- *Slides placed on histological staining tray for individual application and washing.*
- *Post fix in **methanol at -20°C** – 6 mins.*

- *Antigen retrieval:*
  i. *Transfer slides to rack with spaces between slides.*
  ii. *Warm up Antigen Retrieval Buffer in pressure cooker inside microwave for 1 min full power (800W).*
  iii. *Remove Antigen Retrieval Buffer.*
  iv. *Immerse slide rack in Antigen Retrieval Buffer.*
  v. *Microwave for 5 mins full power.*
  vi. *Remove and let cool for 15 mins in sink. Release pressure, remove rack, and continue.*

- *Immunofluorescent Staining*
  i. *Ring sections on slide with Hydrophobic 'PAP' pen.*
  ii. *PBST wash – 3 x 2 mins.*
  iii. *DAKO Protein BLOCK X090930 – 15 mins - ~80µl/slide – Room Temperature (RT).*

- PRIMARY ANTIBODIES - [75µl / section in dilutions as follows]
Diluent – DAKO Ab Diluent s202230

iv. *Pax7 (1:6)*

v. *Incubate $^{I}\alpha$ – +4°C – overnight*

vi. *PBST – 3 x4 mins*
vii. *DAKO Protein BLOCK – 15 mins - ~80µl/slide – Room Temperature (RT).*

- SECONDARY ANTIBODIES - [75µl / section in dilutions as follows]
Diluent – DAKO Ab Diluent s202230

viii. *DαM TRITC: (1:500)*

ix. *Incubate $^{II}\alpha$ – +37°C – 60mins *DARK**

x. *PBST – 3 x4 mins *DARK**
xi. *PBS – 4 mins *DARK**

- *Mount with coverslip:*
  i. *Remove excess wash with kimwipe paper towel.*
  ii. *Add small drop of Vectashield with DAPI soft-mountant on to section.*
  iii. *Apply coverslip. Seal with transparent, translucent nail polish.*
  iv. *Leave to dry in hood before examining under microscopy.*

END

MyH7 Immunofluorescence Stain for human type 1 skeletal muscle fibres

Summary

Immunofluorescence stain to identify muscle heavy chain 7 (MyH7) representing type 1 skeletal muscle fibres in human muscle tissue. Secondary fluorophore labelling provides identification in the TRITC channel.

Equipment	Instruments		Materials
• Slide staining rack • Timer • Dry incubator set to 37°C.	Disposable forceps Paper towel roll Coverslips appropriate to size of sample.	X1 X1 X*n*	• MyH7 (SC53090) primary antibody (Santa Cruz) • Donkey anti-mouse TRITC (PA128625) (Fisher Scientific) • Vectashield with DAPI (H-1200) (Vector Labs)

Solution(s)		**Method**
0.05% PBST		Add 500µl of Tween 20 to 1L of X1 PBS.
Phosphate Buffered Saline X1	1000ml	
Tween 20	500µl	

Methods

A. Prepare solutions.

- *See 'Solutions' section for more detail.*
- *Antigen retrieval buffer can be made in advance.*

B. Sectioning.

- *Cut tissue – cryostat (snap frozen – be careful with thawing).*
- *Superfrost Plus slides preferred to prevent sections lifting during staining protocol.*
- *Label slides with standard format in pencil: Date, Name, Experiment, Sample number etc.*

Staining.

- *Rack up slides to be stained.*
- *Slides placed on histological staining tray for individual application and washing.*
- *Post fix in **methanol at -20°C** – 6 mins.*

- *Immunofluorescent Staining*

i. Ring sections on slide with Hydrophobic 'PAP' pen.
ii. PBST wash – 3 x 2 mins.
iii. DAKO Protein BLOCK X090930 – 15 mins - ~80µl/slide – Room Temperature (RT).

- PRIMARY ANTIBODIES - [75µl / section in dilutions as follows]
Diluent – DAKO Ab Diluent s202230
iv. MyH7 (1:500)

v. Incubate lα – +4°C – overnight

vi. PBST – 3 x4 mins
vii. DAKO Protein BLOCK – 15 mins - ~80µl/slide – Room Temperature (RT).

- SECONDARY ANTIBODIES - [75µl / section in dilutions as follows]
Diluent – DAKO Ab Diluent s202230
viii. DαM TRITC: (1:750)

ix. *Incubate $^{II}\alpha$ – +37°C – 60mins *DARK**

x. *PBST – 3 x4 mins *DARK**
xi. *PBS – 4 mins *DARK**

- *Mount with coverslip:*
  i. *Remove excess wash with kimwipe paper towel.*
  ii. *Add small drop of Vectashield with DAPI soft-mountant on to section.*
  iii. *Apply coverslip. Seal with transparent, translucent nail polish.*
  iv. *Leave to dry in hood before examining under microscopy.*

END

Flow cytometry protocol for human skeletal muscle cells

Summary

Flow cytometry procedure for identification of human muscle tissue.

Read all MSDS, Risk Assessment, and be aware of PPE and mechanical controls for this protocol before beginning.

Methods

A. Prepare solutions.
   - *See 'Solutions' section for more detail.*
   - *Antigen retrieval buffer can be made in advance.*

B. Thaw muscle sample and preparation for digestion.
   - *Thaw sample to room temperature (RT).*
   - *Human skeletal muscle tissue sample size ~3mm^3.*
   - *Trim sample of excess fat, tendon, connective tissue, fascia – and mechanically mince with scalpel.*

C. Sample Digestion
   - *Add 0.1% Collagenase XI II (1mg/ml) in DMEM with high glucose, 10% FBS, 1% Pen/Strep for 70 mins at +37°C.*
   - *Intermittent slow manual needle trituration (18G).*
   - *Spin 700rpm/250g for 5 mins.*
   - *Discard supernatant.*

D. Wash and Re-Suspend Digest in PBS
   - *Add 1X PBS to resuspend.*
   - *Spin 700rpm/250g for 5 min.*
   - *Discard supernatant.*

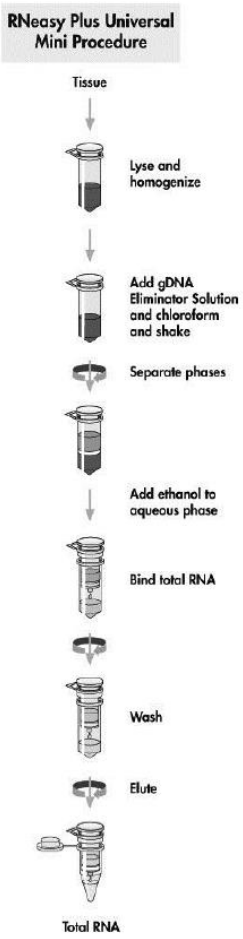
E. Resuspend in PBS.
   - *Add 1X PBS to resuspend.*
   - *Chill Eppendorf at +5°C on wet ice.*

F. Check for Live/Dead stain with Propidium Iodide.
   - *Add Propidium Iodide.*
   - *Run simple gating strategy on flow cytometer. [Performed by facility technicians].*

END

RNeasy Plus Universal Mini Kit – RNA Extraction Method

RNeasy Plus Universal Mini Procedure:



Reagents / Equipment required:

- Chloroform
- Ethanol (70% and 96–100% - 'molecular grade')[not denatured]
- Sterile, RNase-free pipet tips
- For stabilization of RNA in tissues: liquid nitrogen and dry ice
- 1.5 ml or 2 ml microcentrifuge tubes
- Microcentrifuge(s) (with rotor for 2 ml tubes) for centrifugation at 4°C and at room temperature (15–25°C)
- Equipment for tissue disruption and homogenization

Starting material: No more than 30mg tissue.

Prep materials:

- All autoclaved consumables ready.
  - (Tips, eppendorfs; 0.5, 1.5, 2.0ml, homogenizer tips).
- Dry ice bucket with stored samples.
- Wet ice bucket.
- All QIAGEN kit contents ready and laid out. Add EtOH for first use.

Step	Instruction	Technical notes
1	Remove tissue from storage	
2	Cut up tissue if too large	30mg maximum, will clog otherwise and vastly reduce yield and quality. Don't let thaw. Disposable scalpel on dry ice cooled glass tray and cut into small pieces.
3	Disrupt and homogenize tissue: - Place into 900ul QIAzol - Use fresh homogenizer tip until uniformly homogenous.	Homogenize on ice in separate ice bucket so 'splash' doesn't contaminate other samples in run.
4	Incubate the homogenate at room temp (RT) for **5 mins**	
5	Add 100ul gDNA Eliminator Solution - Shake **15 secs**	
6	Add 180ul chloroform - Shake **15 secs**	
7	Incubate the homogenate at room temp (RT) for **3 mins**	
8	Centrifgue at 12000g for 15 mins at 4°C	
	Continued on next page..	

After centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. For tissues with an especially high fat content, an additional, clear phase may be visible below the red, organic phase. The volume of the aqueous phase should be approximately 600 µl.

9. Transfer the upper, aqueous phase (usually 600 µl) to a new microcentrifuge tube (not supplied).

10. Add 1 volume (usually 600 µl) of 70% ethanol, and mix thoroughly by pipetting up and down. Do not centrifuge. Proceed immediately to step 11.

    Note: The volume of lysate may be less than 600 µl due to loss during homogenization and centrifugation.

    Precipitates may be visible after addition of ethanol. Resuspend precipitates completely by vigorous shaking, and proceed immediately to step 11.

11. Transfer up to 700 µl of the sample to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) at room temperature (15–25°C). Discard the flow-through.*

    Reuse the collection tube in step 12.

12. Repeat step 11 using the remainder of the sample. Discard the flow- through.*

    Reuse the collection tube in step 13.

13. Add 700 µl Buffer RWT to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the membrane. Discard the flow-through.*
    Reuse the collection tube in step 14.

    After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.*

    Note: Buffer RWT is supplied as a concentrate. Ensure that ethanol is added to Buffer RWT before use (see “Things to do before starting”, page 17).

14. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the membrane. Discard the flow-through.
    Reuse the collection tube in step 15.

    Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see “Things to do before starting”, page 17).

15. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the membrane.

    The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

    Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

16. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow- through. Close the lid gently, and centrifuge at full speed for 1 min.

    Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 15.

17. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently. To elute the RNA, centrifuge for 1 min at ≥8000 x g (≥10,000 rpm).
18. Repeat step 17 using another volume of RNase-free water, or using the eluate from step 17 (if high RNA concentration is required). Reuse the collection tube from step 17.

    If using the eluate from step 17, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

    END

Trizol – RNA Extraction Method

Working with RNA:

- Always use gloves
- Before starting, clean all working areas and tools (pipettes, forcepts, bottles etc) with 70/75% EtOH.and Trigene.
- Autoclave all necessary consumables (tips etc.) 1-2 days in advance.
- Always use filtertips for pipetting and be sure to wlays change tips between each sample.
- Do not have RNA samples at room temperature for too long (except when they are in Trizol). Always have the tubes on wet ice, then dry ice before storage.
- Be paranoid, RNases are everywhere – especially on skin to protect you from viruses.

RNA protocol background:

Trizol Reagent: A mono-phasic solution of phenol and gunanidine isothiocynate that disrupts cells and dissolves cell components while maintaining RNA integrity. Working with this solution can be carried out in 15-30°C.

Chloroform: Used for phase separation into 3 phases: aqueous phase (containing RNA), inter-phase (containing protein) and organic phase (containing DNA).

2-propanol/isopropanol: Use for precipitating the RNA from the acquired aqueous phase.

75% EtOH: Used for washing the RNA pellet from phenol and isopropanol residues.

Materials:

- Scalpel per sample
- Well plates or similar sterile area that can be chilled on dry ice during cutting up sample
- Tips – P1000, P200, P10
- Pipettes, gloves, lab coat etc.
- Vortex
- Centrifuge that can be cooled to 4°C
- Ice buckets – one for wet ice, one for dry ice.
- Eppendorf holder racks – one for bench, one for freezer (if doing o/n precipitation)

Reagents:

- RNAse free water (‘molecular grade’)
- Trizol (Life Tech/Thermo Fisher – 15596026)
- Chloroform
- Isopropanol molecular grade (Fisher Scientific – BP2618)
- Molecular grade ethanol

Make up:

- 75% EtOH

RNA Extractions Procedure:

1. Pre-cooling steps
   - Switch on cooling centrifuge so can reach target temperature.
   - Put all sterile cutting tools on dry ice (in packaging) so can reach low temp and not prematurely thaw sample.

2. Homogenization
   - Collect samples. Approximate to similar sizes. Keep on dry ice.
   - Cut up samples as small as possible using scalpel. Be aware that sample fragments may contaminate neighbouring areas. Transfer to 2ml sterile Eppendorf.
   - Different tools for each sample.
   - Transfer samples in eppendorfs to wet ice bucket.
   - Add 1ml of Trizol to the preweighed frozen sample and homogenize it directly for 1-2 mins. (Keep the first samples on ice while working through next coming samples).
   - Phenol waste into separate purple bin.
   - Incubate the sample(s) at room temperature for 5 mins. Triturate 15-20x while incubating.

3. Phase separation
   - Add 200ul chloroform (1:5), cap the tube securely and shake the tube vigourously for at least 15 secs. Incubate the sample at room temperature for 3 mins.
   - Centrifuge the sample at 12000g for 15 mins at 4°C.

4. Precipitation
   - Carefully move the aqueous phase (about 600ul to a clean 1.5ml Eppendorf tube and add 500ul isopropanol.
   - Can also remove protein phase for later analysis.
   - Invert and vortex the sample briefly. *[Now leave at RT for 10 mins if going to incubate o/n]*

Can now leave overnight or while preparing other samples at -20°C. Extending the precipitation stage can help increase RNA yield.

   - Put 75% EtOH and RNase free water on Dry/Wet ice so cold for later steps.
   - Incubate it at room temperature for 10 mins.
   - Centrifuge the sample at 12000g for 10 mins at 4°C. Place and remove the Eppendorf carefully orientated so aware of location of pellet.

5. RNA wash
   - Pour out the suspension in one quick inversion without losing the pellet. Can hold upside down while drip dries but do not shake. Dab on kim-wipe.

- Add 1ml of cold 75% EtOH cooled on wet ice.
- Vortex the sample briefly so the pellet is dislodged and centrifuge at 7500g for 5 mins at 4°C.

6. Dissolving RNA:
    - Pour out the suspension in one quick inversion without losing the pellet.
    - Air-dry the pellet for 2-15 min to completely remove remaining EtOH. Can also use kim-wipe to absorb EtOH from inside Eppendorf. Do not touch near or on pellet.
    - Add 30ul of DEPC/RNase free cold water and dissolve the pellet by triturating and vortexing.
    - Keep on wet ice.
    - Analyse with Nanodrop if required. Record ratios and weight yield.
    - Store at -80°C.

END

NanoDrop ND-1000 Protocol

Operation
A 1 ul sample is pipetted onto the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. The gap is controlled to both 1mm and 0.2 mm paths. A pulsed xenon flash lamp provides the light source and a spectrometer utilizing a linear CCD array is used to analyze the light after passing through the sample. The instrument is controlled by PC based software, and the data is logged in an archive file on the PC.

TapeStation 2200 Protocol

Agilent RNA ScreenTape System Quick Guide

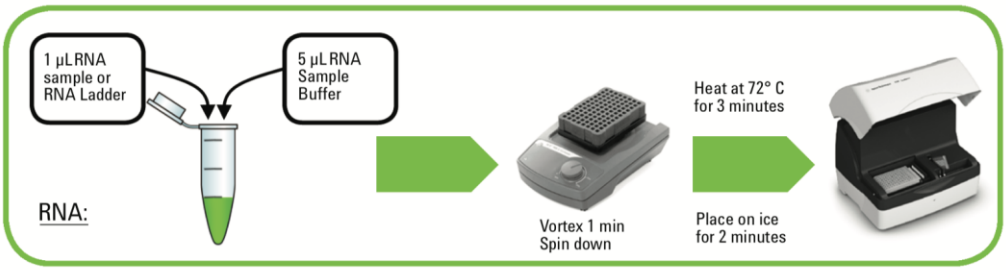
Sample Preparation RNA ScreenTape Assay

Parts required	p/n	Description
	5067-5577	RNA ScreenTape Sample Buffer
	5067-5578	RNA ScreenTape Ladder

1. Allow reagents to equilibrate at room temperature for 30 min
2. Vortex mix before use
3. Thaw total RNA samples on ice
4. If running ladder, prepare by mixing 5 µL RNA Sample Buffer ( ) with 1 µL RNA Ladder ( ).
5. Prepare sample by mixing 5 µL RNA Sample Buffer ( ) with 1 µL RNA sample.

NOTE For best results, use the reverse pipetting technique.

6. Spin down, then vortex using IKA vortexer and adaptor at 2000 rpm for 1 min.
7. Spin down to position the sample at the bottom of the tube.
8. Ladder/Sample denaturation
   a. Heat ladder and samples at 72 °C (162 °F) for 3 min
   b. Place ladder and samples on ice for 2 min
   c. Spin down to position the sample at the bottom of the tube



Sample Analysis
1. Load samples into the 2200 TapeStation instrument.
2. Select the required samples on the 2200 TapeStation Controller Software.
3. Click **Start** and specify a filename with which to save your results.

END

Genomic DNA Elimination Protocol

(Protocol from Primer Design DNase kit – PrimerDesign Handbook HB12.01.03)

Starting material:

- RNA samples (e.g. from Trizol extactions)
- Primer Design DNase kit

Methods:

1. Pre-steps
   - Sterilise all necessary equipment.
   - Switch on heat blocks so can reach target temperature.
     i. One to 30°C, one to 55°C.
     ii. Check temperature with thermometer or probe.
   - Remove kit from freezer and leave on wet ice to warm up.
   - Remove samples from -80°C freezer and leave on wet ice to warm up.

Kit to be used in following ratio:
5µl 10X Precision DNase reaction buffer for every 50µl of RNA
1µl Precision DNase enzyme can eliminate DNA from up to 100µl of RNA solution

2. Combine 10X Precision DNase reaction buffer with Precision DNase enzyme in Eppendorf.

3. Add to each sample, triturate briefly and vortex briefly

4. Incubate for 10-30 [**15**] minutes at 30°C. (DNase Treatment)
   - Longer than 30 minutes degrades RNA

5. Incubate for 5 minutes at 55°C. (DNase inactivation)

6. Transfer to wet ice

7. Proceed to Reverse Transcription step or store DNase treated RNA at < -20°C **[-80°C**] for later use

END

Reverse Transcription Protocol

(Protocol from Thermo Fisher Scientific High Capacity cDNA Reverse Transcription kit (4368814))

Contents and storage

Contents	Cat. Nos. 4368813 and 4374967	Cat. Nos. 4368814 and 4374966	Storage
10X RT Buffer, 1.0 mL	2 tubes	1 tube	−25°C to −15°C
10X RT Random Primers, 1.0 mL	2 tubes	1 tube	
25X dNTP Mix (100 mM)	1 tube, 1.0 mL	1 tube, 0.2 mL	
MultiScribe™ Reverse Transcriptase, 50 U/µL	1 tube, 1.0 mL	2 tubes, 0.1 mL	
RNase Inhibitor, 100 µL [1]	10 tubes	2 tubes	

[1] Included in Cat. Nos. 4374966 and 4374967only.

Workflow

Prepare 2X reverse transcription master mix
▼
Add RNA to reverse transcription reactions
▼
Perform reverse transcription in a thermal cycler
▼
Use the reverse transcription reactions (cDNA) directly for quantitative or other PCR applications

▼
Store the reverse transcription reactions (cDNA) at:
- 2°C to 6°C for short-term storage
- −25°C to −15°C for long-term storage

Reverse transcription reaction guidelines

The kit contains reagents that, when combined, form a 2X reverse transcription (RT) master mix. An equal volume of RNA sample should be added. To avoid RNase contamination, RNase-free reagents and consumables must be used.

Prepare the 2X RT master mix

1. Allow the kit components to thaw on ice.
2. Calculate the volume of components needed to prepare the required number of reactions.

Note: Prepare the RT master mix on ice.

Component	Volume	
	With RNase Inhibitor	Without RNase Inhibitor
10X RT Buffer	2.0 µL	2.0 µL
25X dNTP Mix (100 mM)	0.8 µL	0.8 µL
10X RT Random Primers	2.0 µL	2.0 µL
MultiScribe™ Reverse Transcriptase	1.0 µL	1.0 µL
RNase Inhibitor	1.0 µL	—
Nuclease-free H_2O	3.2 µL	4.2 µL
Total per reaction	10.0 µL	10.0 µL

IMPORTANT! Include additional reactions in the calculations to provide excess volume for the loss that occurs during reagent transfers.

3. Place the 2X RT master mix on ice and mix gently.

Prepare the reverse transcription reactions

1. Pipette 10 µL of 2X RT master mix into each well of a 96-well reaction plate or individual tube.
2. Pipette 10 µL of RNA sample into each well, pipetting up and down two times to mix.
3. Seal the plates or tubes.
4. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.
5. Place the plate or tubes on ice until you are ready to load the thermal cycler.

Program the thermal cycling conditions

Program the thermal cycler using the conditions below.

IMPORTANT! These conditions are optimized for use with the High-Capacity cDNA Reverse Transcription Kits.

Settings	Step 1	Step 2	Step 3	Step 4
Temp.	25°C	37°C	85°C	4°C
Time	10 minutes	120 minutes	5 minutes	∞

```
Instrument Name: 2990225810
Initiating Hub: 2990225810
Run Method: high capacity cdna RT-APPLIED BIOSYST
EM
Run ID: 201811070224
User Name: None
Reaction Volume (uL): 20
Cover Heating: On
Cover Temperature (Degrees C): 105.0
Notes: MG
Method:
[Stage 1] x1
1) 25.0deg C, 10:00(mm:ss), 100%
[Stage 2] x1
1) 37.0deg C, 60:00(mm:ss), 100%
[Stage 3] x1
1) 37.0deg C, 60:00(mm:ss), 100%
[Stage 4] x1
1) 85.0deg C, 5:00(mm:ss), 100%
[Stage 5] x1
1) 4.0deg C, Infinite, 100%
```

END

Quantitative Polymerase Chain Reaction

GeNorm Reference Gene Selection Protocol – Methodology Development

(From: PrimerDesign GeNorm SybrGreen Handbook HB01.02.07)

Bench-side Protocol

To minimise the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR cabinet. Filter tips are recommended for all pipetting steps

1. **Pulse-spin each tube in a centrifuge before opening.**
   This will ensure lyophilised primer mix is in the base of the tube and is not spilt upon opening the tube.

2. **Resuspend lyophilised primer mix in RNAse/DNAse free water provided.**

   To ensure complete resuspension, vortex each tube thoroughly, allow to stand for 5 minutes and vortex again before use.

Component	Volume
Primer mix (BLUE)	220 µl

There is a 10% over pipette in each kit

3. **When using Primerdesign 2x *PrecisionPLUS™* or *PrecisionFAST™* qPCR Mastermix, make up a mix for each reference gene according to the protocol below.**

Component	1 Reaction
Resuspended primer mix(BLUE)	1 µl*
Primerdesign 2x *PrecisionPLUS™/PrecisionFAST™* Mastermix	10 µl
RNAse/DNAse free water (WHITE)	4 µl
Final volume	**15 µl**

*working concentration of primers = 300nM in a 20µl reaction

4. **Pipette 15µl of the mix into each well according to your plate set up.**

   All samples for each reference must be run on the same plate. However, different reference genes may be run on separate plates. If using multiple plates, pour all plates on the same occasion. Run all data points in duplicate wells.

	Reference Genes											
	ACTB				UBC				GAPDH			
cDNA samples	1	1	9	9	1	1	9	9	1	1	9	9
	2	2	10	10	2	2	10	10	2	2	10	10
	3	3	11	11	3	3	11	11	3	3	11	11
	4	4	12	12	4	4	12	12	4	4	12	12
	5	5	13	13	5	5	13	13	5	5	13	13
	6	6	14	14	6	6	14	14	6	6	14	14
	7	7	15	15	7	7	15	15	7	7	15	15
	8	8	Water	Water	8	8	Water	Water	8	8	Water	Water

Example plate layout for a geNorm analysis using 15 cDNA samples.

5. **Prepare 66µl (6 gene kit) or 132µl (12 gene kit) of cDNA for each sample at a concentration of 5ng/µl in RNAse/DNAse free water.**
   These suggested volumes include an additional 10% more than required to allow for pipette calibration inaccuracies. If the concentration of cDNA is not known, then dilute your RT reactions 1:10 (10µl of RT and 90µl of water). Ensure you have high quality cDNA before proceeding.

6. **Pipette 5µl of diluted cDNA into each well of your 96-well plate according to your plate layout .**
   The final volume in each well is 20µl.

Amplification Protocol

1. Amplification conditions using Primerdesign 2x *PrecisionPLUS*™ qPCR Mastermix .

	Step	Time	Temp
	Enzyme activation	2min	95°C
Cycling x40	Denaturation	10s	95°C
	DATA COLLECTION*	60s	60°C
	Melt Curve**		

*Fluorogenic data should be collected during this step through the SYBR® green channel.
**A post PCR run melt curve can be used to prove the specificity of the primers. See the manufactures instructions for your hardware platform

END

(qPCR protocol from PrimerDesign PrecisionPLUS qPCR – Handbook HB04.13.02)

Bench-side protocol

When using Primerdesign kits:

For each 20µl real-time PCR reaction add the following to each reaction tube

Components	1 Reaction
PrecisionPLUS qPCR Master Mix	10 µl
Primer/Probe mix	1 µl
Template (25ng)	5 µl
RNase/DNase free water	4 µl
Final volume	**20 µl**

Amplification protocols

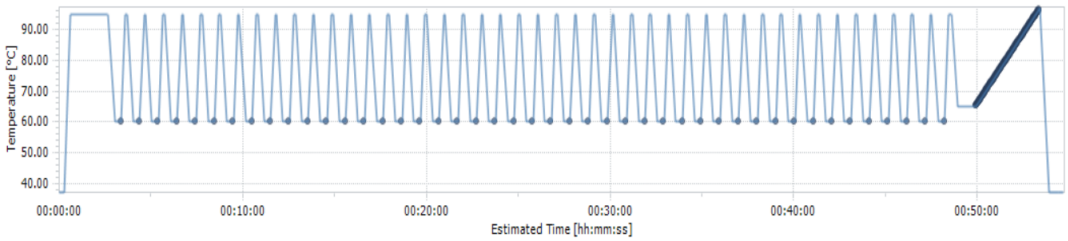
Precision®PLUS Master Mix

For use with SYBR®green gene detection kits

	Step	Time	Temp
	Enzyme activation – Hot Start	2min	95°C
Cycling x40***	Denaturation	10s	95°C
	DATA COLLECTION*	60s	60°C
	Melt Curve**		

*Fluorogenic data should be collected during this step through the SYBR®green channel.
**A post PCR run melt curve can be used to prove the specificity of the primers. See the manufactures instructions for your hardware platform
*** For low copy number targets, giving late detection, a further 10 cycles may be needed to generate the complete amplification plot

(LightCycler 96 Program Output)



Instrument Serial Number: 000000000010599
Instrument Software Version: 1.01.00.0045
Application Software Version:
Plate Id:
Creation Date:
Run Start Date:
Run End Date:

Run Definition
No Of Channels: 1
Integration Time Mode: Dynamic
Channel: 470/514
Dye: SYBR Green I
Quant Factor: 20.00
Melt Factor: 1.20

Programs

Program Name: Preincubation
No Of Cycles: 1
Steps

Measurement: None	Duration [s]: 120	Ramp: 4.4	TargetTemperature: 95	

Program Name: 2 Step Amplification
No Of Cycles: 45
Steps

Measurement: None	Duration [s]: 5	Ramp: 4.4	TargetTemperature: 95	
Measurement: Single	Duration [s]: 20	Ramp: 2.2	TargetTemperature: 60	

Program Name: Melting
No Of Cycles: 1
Steps

Measurement: None	Duration [s]: 10	Ramp: 4.4	TargetTemperature: 95	
Measurement: None	Duration [s]: 60	Ramp: 2.2	TargetTemperature: 65	
Measurement: Continuous	Duration [s]: 1	Ramp: 0.2	TargetTemperature: 97	Acqs: 5

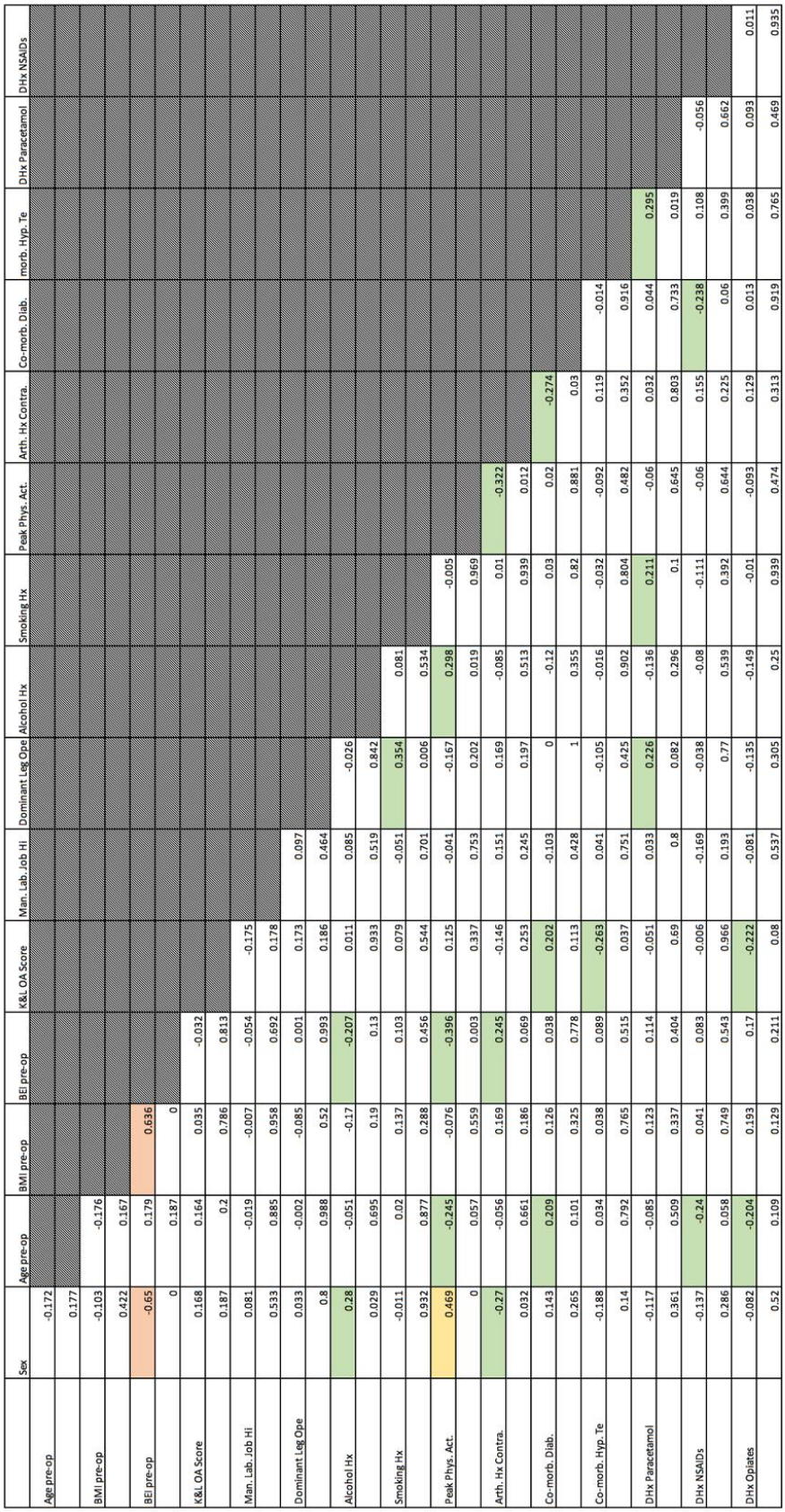
Program Name: Cooling
No Of Cycles: 1
Steps

Measurement: None	Duration [s]: 30	Ramp: 2.2	TargetTemperature: 37

Appendix C: Additional Data

Correlation Matrices

Demographic and Clinical Baseline Factor Correlations



	Sex	Age pre-op	BMI pre-op	BEI pre-op	K&L OA Score	Man. Lab. Job Hi	Dominant Leg Ope	Alcohol Hx	Smoking Hx	Peak Phys. Act.	Arth. Hx Contra.	Co-morb. Diab.	morb. Hyp. Te	DHx Paracetamol	DHx NSAIDs
Age pre-op	-0.172														
	0.177														
BMI pre-op	-0.103	-0.176													
	0.422	0.167													
BEI pre-op	-0.65	0.179	0.636												
	0	0.187	0												
K&L OA Score	0.168	0.164	0.035	-0.032											
	0.187	0.2	0.786	0.813											
Man. Lab. Job Hi	0.081	-0.019	-0.007	-0.054	-0.175										
	0.533	0.885	0.958	0.692	0.178										
Dominant Leg Ope	0.033	-0.002	-0.085	0.001	0.173	0.097									
	0.8	0.988	0.52	0.993	0.186	0.464									
Alcohol Hx	0.28	-0.051	-0.17	-0.207	0.011	0.085	-0.026								
	0.029	0.695	0.19	0.13	0.933	0.519	0.842								
Smoking Hx	-0.011	0.02	0.137	0.103	0.079	-0.051	0.354	0.081							
	0.932	0.877	0.288	0.456	0.544	0.701	0.006	0.534							
Peak Phys. Act.	0.469	-0.245	-0.076	-0.396	0.125	-0.041	-0.167	0.298	-0.005						
	0	0.057	0.559	0.003	0.337	0.753	0.202	0.019	0.969						
Arth. Hx Contra.	-0.27	-0.056	0.169	0.245	-0.146	0.151	0.169	-0.085	0.01	-0.322					
	0.032	0.661	0.186	0.069	0.253	0.245	0.197	0.513	0.939	0.012					
Co-morb. Diab.	0.143	0.209	0.126	0.038	0.202	-0.103	0	-0.12	0.03	0.02	-0.274				
	0.265	0.101	0.325	0.778	0.113	0.428	1	0.355	0.82	0.881	0.03				
Co-morb. Hyp. Te	-0.188	0.034	0.038	0.089	-0.263	0.041	-0.105	-0.016	-0.032	-0.092	0.119	-0.014			
	0.14	0.792	0.765	0.515	0.037	0.751	0.425	0.902	0.804	0.482	0.352	0.916			
DHx Paracetamol	-0.117	-0.085	0.123	0.114	-0.051	0.033	0.226	-0.136	0.211	-0.06	0.032	0.044	0.295		
	0.361	0.509	0.337	0.404	0.69	0.8	0.082	0.296	0.1	0.645	0.803	0.733	0.019		
DHx NSAIDs	-0.137	-0.24	0.041	0.083	-0.006	-0.169	-0.038	-0.08	-0.111	-0.06	0.155	-0.238	0.108	-0.056	
	0.286	0.058	0.749	0.543	0.966	0.193	0.77	0.539	0.392	0.644	0.225	0.06	0.399	0.662	
DHx Opiates	-0.082	-0.204	0.193	0.17	-0.222	-0.081	-0.135	-0.149	-0.01	-0.093	0.129	0.013	0.038	0.093	0.011
	0.52	0.109	0.129	0.211	0.08	0.537	0.305	0.25	0.939	0.474	0.313	0.919	0.765	0.469	0.935

Cell Contents:	Spearman rho correlation
	P-Value

Functional Metric Correlations - All Time-points

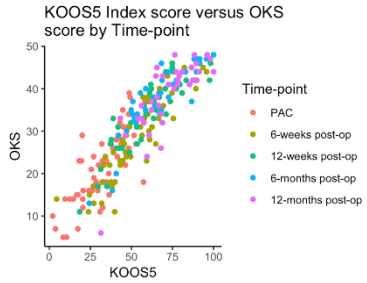
		PAC			6-weeks post-op			12-weeks post-op			6-months post-op			12-months post-op		
		Leg Power Ratio	ALF Score	Daily Step Count	Leg Power Ratio	ALF Score	Daily Step Count	Leg Power Ratio	ALF Score	Daily Step Count	Leg Power Ratio	ALF Score	Daily Step Count	Leg Power Ratio	ALF Score	Daily Step Count
ALF Score		-0.499			-0.474			-0.626			-0.591			-0.718		
		0			0.002			0			0.001			0		
Daily Step Count		0.104	-0.324		0.038	-0.331		0.294	-0.345		0.374	-0.623		0.229	-0.432	
		0.607	0.099		0.846	0.08		0.164	0.099		0.066	0.001		0.272	0.031	
ROM		0.398	-0.365	-0.184	0.47	-0.293	0.058	0.594	-0.544	0.145	0.539	-0.476	0.165	0.509	-0.572	-0.119
		0.002	0.006	0.358	0.003	0.067	0.767	0.001	0.003	0.498	0.003	0.009	0.42	0.002	0	0.571

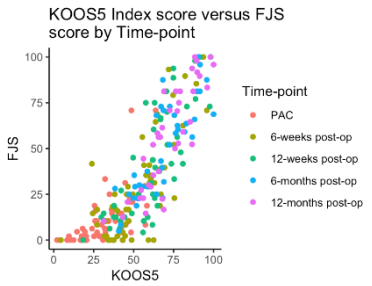
Cell Contents:	Pearson correlation
	P-Value

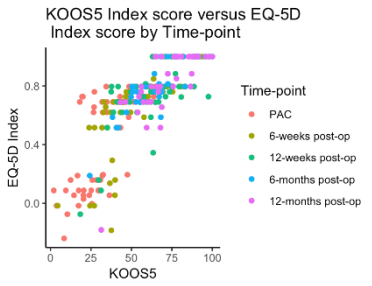
PROM Results Correlation Assessments

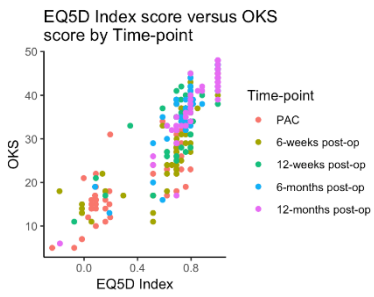
		PAC			6-weeks post-op			12-weeks post-op			6-months post-op			12-months post-op		
		KOOS5 Index	OKS	FJS	KOOS5 Index	OKS	FJS	KOOS5 Index	OKS	FJS	KOOS5 Index	OKS	FJS	KOOS5 Index	OKS	FJS
OKS	KOOS5 Index	0.791			0.897			0.891			0.871			0.803		
	OKS	0			0			0			0			0		
	FJS	0.593	0.586		0.756	0.724		0.785	0.758		0.83	0.808		0.863	0.817	
	EQ-5D Index	0	0	0.001	0	0	0	0	0	0	0	0	0	0	0	0
FJS	KOOS5 Index	0.686	0.781	0.409	0.738	0.775	0.548	0.656	0.772	0.566	0.836	0.888	0.786	0.744	0.908	0.718
	OKS	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	FJS	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

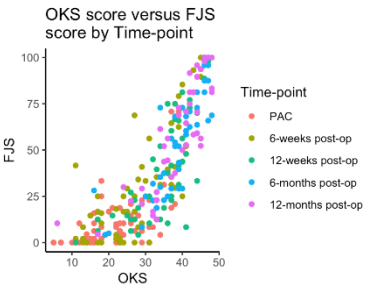
Cell Contents:	
Pearson correlation	
P-Value	

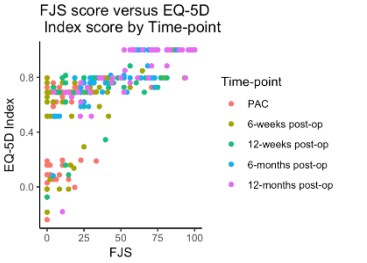












Biometric Data and Functional Outcome Matrices

Comparison of 12-week post-op Sleep Data and Functional Outcomes

	KOOS5 Index (12W)	Leg Power Ratio (NLR 12W)	ALF Score (12W)	Daily Step Count (12W)
Total Sleep at 12 weeks post-op	-0.380906	-0.278152	0.446539	-0.490387
	0.0663	0.1882	0.0287	0.0150
Total Light Sleep at 12 weeks post-op	0.234888	0.036572	0.102528	-0.023157
	0.2692	0.8653	0.6336	0.9145
Total Deep Sleep at 12 weeks post-op	-0.141470	0.228142	-0.175680	-0.106889
	0.5096	0.2836	0.4116	0.6191
Total Light Sleep/ Total Deep Sleep at 12 weeks post-op	0.205182	-0.104914	0.101075	-0.019747
	0.3361	0.6256	0.6384	0.9270

Comparison of pre-op ROM and Functional Outcomes

	KOOS5 Index (PAC)	Leg Power Ratio (NLR PAC)	ALF Score (PAC)	Daily Step Count (PAC)
Fixed Flexion Pre-Op	-0.168394	-0.09505	0.007118	0.359116
	0.2148	0.4819	0.9585	0.0658
Fixed Flexion 12-months Post-Op	-0.057665	-0.18515	0.186885	0.076558
	0.7421	0.287	0.2899	0.7101
Flexion <90 degrees Pre-Op	-0.00512	-0.108214	0.282523	0.050123
	0.9699	0.423	0.0349	0.8039
Flexion <90 degrees 12-months post-op	-0.37599	-0.308164	0.209054	0.011066
	0.026	0.0717	0.2354	0.9572

Multivariate Regression Raw Model Outputs

Baseline Functional and PROM Contributing Factors to Surgical Outcomes

12-week Leg Power

```
Call:
lm(formula = datTert$Leg.Power.Ratio..NLR.12W. ~ datTert$NLR_RatPAC +
    datTert$ALF_PAC + datTert$KOOS5_PAC + datTert$EQ5D_lnPAC)
Residuals:
     Min       1Q   Median       3Q      Max
-0.49622 -0.16336 -0.01235  0.13871  0.41386
Coefficients:
                    Estimate Std. Error t value Pr(>|t|)
(Intercept)           1.7305   0.295723   5.852 1.23E-05
datTert$NLR_RatPAC    0.5653   0.142492   3.968 0.000824
datTert$ALF_PAC      -0.0270   0.006843  -3.947 0.000866
datTert$KOOS5_PAC    -0.0191   0.007137  -2.677 0.014916
datTert$EQ5D_lnPAC    0.6305   0.296622   2.126 0.046874
Residual standard error: 0.2526 on 19 degrees of freedom
  (39 observations deleted due to missingness)
Multiple R-squared:  0.7737,    Adjusted R-squared:  0.726
F-statistic: 16.24 on 4 and 19 DF,  p-value: 6.195e-06
```

12-month Leg Power

```
Call:
lm(formula = datTert$Leg.Power.Ratio..NLR.12M. ~ datTert$NLR_RatPAC +
    datTert$KOOS5_PAC + datTert$EQ5D_lnPAC)
Residuals:
     Min       1Q   Median       3Q      Max
-0.90774 -0.16222  0.03238  0.28505  0.65083
Coefficients:
                    Estimate Std. Error t value Pr(>|t|)
(Intercept)           0.9043   0.231076   3.913 0.000556
datTert$NLR_RatPAC    1.0681   0.183372   5.825 3.36E-06
datTert$KOOS5_PAC    -0.0223   0.009172  -2.434 0.021849
datTert$EQ5D_lnPAC    1.1896   0.359453   3.309 0.002657
Residual standard error: 0.4128 on 27 degrees of freedom
  (32 observations deleted due to missingness)
Multiple R-squared:  0.626,    Adjusted R-squared:  0.5844
F-statistic: 15.06 on 3 and 27 DF,  p-value: 5.901e-06
```

12-week ALF Timed Functional Score

```
Call:
lm(formula = datTert$ALF.Score..12W. ~ datTert$ALF_PAC + datTert$STEP_PAC +
    datTert$KOOS5_PAC + datTert$EQ5D_lnPAC)
Residuals:
    Min      1Q  Median      3Q     Max
-9.4310 -5.7010 -2.7570  3.3780 24.6400
Coefficients:
                    Estimate Std. Error t value Pr(>|t|)
(Intercept)          -24.510   1.12E+01  -2.182 0.043451
datTert$ALF_PAC        1.260   2.62E-01   4.829 0.000157
datTert$STEP_PAC       0.000   9.35E-04   2.156 0.045745
datTert$KOOS5_PAC      0.680   2.49E-01   2.740 0.013967
datTert$EQ5D_lnPAC   -31.310   1.09E+01  -2.884 0.010305
Residual standard error: 8.836 on 17 degrees of freedom
  (41 observations deleted due to missingness)
Multiple R-squared:  0.6301,    Adjusted R-squared:  0.543
F-statistic: 7.238 on 4 and 17 DF,  p-value: 0.001356
```

12-month ALF Timed Functional Score

Call:
lm(formula = datTert$ALF.Score..12M. ~ datTert$ALF_PAC + datTert$STEP_PAC +
datTert$KOOS5_PAC + datTert$EQ5D_InPAC)
Residuals:

Min	1Q	Median	3Q	Max
-6.817	-3.19	-1.003	1.809	15.386

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-21.66	7.87E+00	-2.753	0.0136
datTert$ALF_PAC	1.17	1.78E-01	6.591	4.58E-06
datTert$STEP_PAC	0.00	6.88E-04	2.122	0.0488
datTert$KOOS5_PAC	0.47	1.72E-01	2.758	0.0134
datTert$EQ5D_InPAC	-18.79	7.40E+00	-2.539	0.0212

Residual standard error: 5.998 on 17 degrees of freedom
(41 observations deleted due to missingness)
Multiple R-squared: 0.7515, Adjusted R-squared: **0.693**
F-statistic: 12.85 on 4 and 17 DF, p-value: 5.358e-05

12-week Daily Step Count

Call:
lm(formula = datTert$Daily.Step.Count..12W. ~ datTert$ALF_PAC +
datTert$KOOS5_PAC + datTert$FJS_PAC)
Residuals:

Min	1Q	Median	3Q	Max
-2815.8	-642.3	96.6	474	3585.1

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	7945.2	1586.59	5.008	7.81E-05
datTert$ALF_PAC	-116.63	44.89	-2.598	0.0176
datTert$KOOS5_PAC	-78.7	30.81	-2.554	0.0194
datTert$FJS_PAC	128.21	24.91	5.146	5.74E-05

Residual standard error: 1541 on 19 degrees of freedom
(40 observations deleted due to missingness)
Multiple R-squared: 0.6249, Adjusted R-squared: 0.5657
F-statistic: 10.55 on 3 and 19 DF, p-value: 0.000264

12-month Daily Step Count

Call:
lm(formula = datTert$Daily.Step.Count..12M. ~ datTert$ALF_PAC)
Residuals:

Min	1Q	Median	3Q	Max
-3124.7	-1595.8	-728.4	884.4	7577.4

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	9217.92	1557.63	5.918	5.90E-06
datTert$ALF_PAC	-168.81	54.09	-3.121	0.00498

Residual standard error: 2423 on 22 degrees of freedom
(39 observations deleted due to missingness)
Multiple R-squared: 0.3068, Adjusted R-squared: 0.2753
F-statistic: 9.738 on 1 and 22 DF, p-value: 0.004979

12-week KOOS5 PROM Score

Call:
lm(formula = datTert$KOOS5.Index..12W. ~ datTert$ALF_PAC + datTert$STEP_PAC +
datTert$FJS_PAC)
Residuals:

Min	1Q	Median	3Q	Max
-32.817	-8.526	-0.22	12.076	22.355

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	98.324381	12.45167	7.896	1.01E-07
datTert$ALF_PAC	-1.190705	0.358201	-3.324	0.00322
datTert$STEP_PAC	-0.003406	0.001595	-2.135	0.04472
datTert$FJS_PAC	0.592219	0.242146	2.446	0.02335

Residual standard error: 15.27 on 21 degrees of freedom
(38 observations deleted due to missingness)
Multiple R-squared: 0.44, Adjusted R-squared: 0.36
F-statistic: 5.5 on 3 and 21 DF, p-value: 0.006001

12-month KOOS5 PROM Score

Call:
lm(formula = datTert$KOOS5.Index..12M. ~ datTert$ALF_PAC)
Residuals:

Min	1Q	Median	3Q	Max
-53.842	-9.595	0.734	16.175	40.504

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	72.3599	7.0675	10.238	5.53E-13
datTert$ALF_PAC	-0.1412	0.205	-0.689	0.495

Residual standard error: 20.61 on 42 degrees of freedom
(19 observations deleted due to missingness)
Multiple R-squared: 0.01116, Adjusted R-squared: -0.01238
F-statistic: 0.4741 on 1 and 42 DF, p-value: 0.4949

Baseline Demographic, Lifestyle, and Comorbidity Contributing Factors to Surgical Outcome

12-week Leg Power

Call:
lm(formula = datReg$Leg.Power.Ratio..NLR.12W. ~ datReg$DHx.NSAIDs +
datReg$Peak.Phys..Act..Tegner)
Residuals:

Min	1Q	Median	3Q	Max
-1.10677	-0.26178	-0.01102	0.35984	0.65815

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.44464	0.2114	2.103	0.0457
datReg$DHx.NSAIDs	0.30715	0.19509	1.574	0.128
datReg$Peak.Phys..Act..Tegner	0.07381	0.02903	2.543	0.0176

Residual standard error: 0.4219 on 25 degrees of freedom
(35 observations deleted due to missingness)
Multiple R-squared: 0.2494, Adjusted R-squared: **0.1893**
F-statistic: 4.153 on 2 and 25 DF, p-value: 0.02772

12-month Leg Power

Call:
lm(formula = datReg$Leg.Power.Ratio..NLR.12M. ~ datReg$DHx.Opiates +
datReg$Alcohol.Hx + datReg$Peak.Phys..Act..Tegner)
Residuals:

Min	1Q	Median	3Q	Max
-1.21201	-0.36588	-0.01828	0.33264	1.46933

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.31956	0.36299	3.635	0.000995
datReg$DHx.Opiates	-0.34643	0.20685	-1.675	0.104037
datReg$Alcohol.Hx	-0.1926	0.12638	-1.524	0.137646
datReg$Peak.Phys..Act..Tegner	0.07894	0.0358	2.205	0.035023

Residual standard error: 0.561 on 31 degrees of freedom
(28 observations deleted due to missingness)
Multiple R-squared: 0.2175, Adjusted R-squared: **0.1418**
F-statistic: 2.873 on 3 and 31 DF, p-value: 0.05211

12-week ALF Timed Functional Score

Call:
lm(formula = datReg$ALF.Score..12W. ~ datReg$DHx.Paracetamol +
datReg$DHx.NSAIDs + datReg$DHx.Opiates)

Residuals:

Min	1Q	Median	3Q	Max
-20.257	-8.355	-0.475	3.149	43.521

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	31.308	5.161	6.067	2.89E-06
datReg$DHx.Paracetamol	-8.488	5.843	-1.453	0.159
datReg$DHx.NSAIDs	-10.979	6.713	-1.635	0.115
datReg$DHx.Opiates	6.989	5.605	1.247	0.224

Residual standard error: 14.31 on 24 degrees of freedom
(35 observations deleted due to missingness)
Multiple R-squared: 0.1674, Adjusted R-squared: **0.06328**
F-statistic: 1.608 on 3 and 24 DF, p-value: 0.2137

12-month ALF Timed Functional Score

Call:
lm(formula = datReg$ALF.Score..12M. ~ datReg$DHx.Opiates + datReg$Man..Lab..Job.Hist. +
datReg$Alcohol.Hx + datReg$SIMD.Quint)

Residuals:

Min	1Q	Median	3Q	Max
-13.837	-5.708	-1.196	5.8	18.715

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	11.751	7.313	1.607	0.1186
datReg$DHx.Opiates	5.533	3.757	1.473	0.1512
datReg$Man..Lab..Job.Hist.	6.878	3.712	1.853	0.0738
datReg$Alcohol.Hx	5.187	2.098	2.472	0.0193
datReg$SIMD.Quint	-2.05	1.384	-1.481	0.149

Residual standard error: 9.409 on 30 degrees of freedom
(28 observations deleted due to missingness)
Multiple R-squared: 0.2796, Adjusted R-squared: **0.1835**
F-statistic: 2.911 on 4 and 30 DF, p-value: 0.03796

12-week Daily Step Count

Call:
lm(formula = datReg$Daily.Step.Count..12W. ~ datReg$Age.Groups +
datReg$SIMD.Quint)

Residuals:

Min	1Q	Median	3Q	Max
-2767.2	-1264	-214	768.9	6425

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	3440.9	1676.8	2.052	0.0528
datReg$Age.Groups	-657.7	261.1	-2.519	0.0199
datReg$SIMD.Quint	850.1	400.2	2.124	0.0457

Residual standard error: 2012 on 21 degrees of freedom
(39 observations deleted due to missingness)
Multiple R-squared: 0.2936, Adjusted R-squared: **0.2263**
F-statistic: 4.363 on 2 and 21 DF, p-value: 0.02602

12-month Daily Step Count

Call:
lm(formula = datReg$Daily.Step.Count..12M. ~ datReg$Co.morb..Hyp..Ten. +
datReg$DHx.Paracetamol + datReg$Smoking.Hx)

Residuals:

Min	1Q	Median	3Q	Max
-3160.9	-1907.5	-453.8	878	6128.6

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	4293	831.2	5.165	4.07E-05
datReg$Co.morb..Hyp..Ten.	-2303.3	1028.3	-2.24	0.036
datReg$DHx.Paracetamol	2975.6	1084.5	2.744	0.0122
datReg$Smoking.Hx	-2307.3	1071.2	-2.154	0.043

Residual standard error: 2366 on 21 degrees of freedom
(38 observations deleted due to missingness)
Multiple R-squared: 0.3697, Adjusted R-squared: **0.2797**
F-statistic: 4.106 on 3 and 21 DF, p-value: 0.01934

12-week KOOS5 PROM Score

Call:
lm(formula = datReg$KOOS5.Index..12W. ~ datReg$K.L.OA.Score +
datReg$Co.morb..Diab. + datReg$Alcohol.Hx)

Residuals:

Min	1Q	Median	3Q	Max
-33.243	-10.967	-1.043	10.407	46.293

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	63.916	8.611	7.423	3.16E-09
datReg$K.L.OA.Score	6.654	3.025	2.199	0.0333
datReg$Co.morb..Diab.	-11.987	7.394	-1.621	0.1123
datReg$Alcohol.Hx	-6.361	2.795	-2.276	0.0279

Residual standard error: 16.26 on 43 degrees of freedom
(16 observations deleted due to missingness)
Multiple R-squared: 0.2009, Adjusted R-squared: **0.1452**
F-statistic: 3.604 on 3 and 43 DF, p-value: 0.02079

12-month KOOS5 PROM Score

Call:
lm(formula = datReg$KOOS5.Index..12M. ~ datReg$Sex + datReg$BMI.Groups +
datReg$K.L.OA.Score + datReg$DHx.Opiates + datReg$Peak.Phys..Act..Tegner)

Residuals:

Min	1Q	Median	3Q	Max
-33.849	-9.291	1.231	10.384	33.339

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	76.129	10.004	7.61	2.30E-09
datReg$Sex	-9.725	5.621	-1.73	0.09113
datReg$BMI.Groups	-3.287	2.221	-1.48	0.146544
datReg$K.L.OA.Score	11.435	3.195	3.579	0.000905
datReg$DHx.Opiates	-7.943	5.309	-1.496	0.142294
datReg$Peak.Phys..Act..Tegner	-1.652	1.007	-1.641	0.108431

Residual standard error: 16.58 on 41 degrees of freedom
(16 observations deleted due to missingness)
Multiple R-squared: 0.3881, Adjusted R-squared: **0.3134**
F-statistic: 5.2 on 5 and 41 DF, p-value: 0.0008715

Baseline Physiological Contributing Factors to Surgical Outcomes

12-week Leg Power

Call:
lm(formula = datReg$Leg.Power.Ratio..NLR.12W. ~ datReg$Fib..Type.Ratio +
datReg$Myf5.Group)
Residuals:

Min	1Q	Median	3Q	Max
-0.84526	-0.36122	-0.01384	0.3204	0.84332

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.0426	0.3441	3.03	0.00719
datReg$Fib..Type.Ratio	-0.3607	0.3472	-1.039	0.31268
datReg$Myf5.Group	0.1816	0.1501	1.21	0.2419

Residual standard error: 0.4967 on 18 degrees of freedom
(42 observations deleted due to missingness)
Multiple R-squared: 0.1244, Adjusted R-squared: **0.02712**
F-statistic: 1.279 on 2 and 18 DF, p-value: 0.3025

12-month Leg Power

Call:
lm(formula = datReg$Leg.Power.Ratio..NLR.12M. ~ datReg$Fib..Type.Ratio +
datReg$MyoD.Group + datReg$CDK2NA.Group)
Residuals:

Min	1Q	Median	3Q	Max
-1.2158	-0.25987	0.05412	0.24891	0.80398

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.4519	0.3869	3.753	0.0011
datReg$Fib..Type.Ratio	-0.9053	0.3532	-2.563	0.01773
datReg$MyoD.Group	0.4709	0.1618	2.911	0.00811
datReg$CDK2NA.Group	-0.4165	0.232	-1.795	0.08639

Residual standard error: 0.5271 on 22 degrees of freedom
(37 observations deleted due to missingness)
Multiple R-squared: 0.3793, Adjusted R-squared: **0.2946**
F-statistic: 4.48 on 3 and 22 DF, p-value: 0.01337

12-week ALF Timed Functional Score

Call:
lm(formula = datReg$ALF.Score..12W. ~ datReg$Myog.Group + datReg$TNF.Group)
Residuals:

Min	1Q	Median	3Q	Max
-13.386	-6.396	-1.657	1.423	41.154

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	23.763	6.095	3.899	0.000772
datReg$Myog.Group	2.28	1.609	1.417	0.170453
datReg$TNF.Group	-4.306	3.563	-1.209	0.239642

Residual standard error: 12.1 on 22 degrees of freedom
(38 observations deleted due to missingness)
Multiple R-squared: 0.1002, Adjusted R-squared: **0.01836**
F-statistic: 1.224 on 2 and 22 DF, p-value: 0.3132

12-month ALF Timed Functional Score

Call:
lm(formula = datReg$ALF.Score..12M. ~ datReg$Fib..Type.Ratio +
datReg$MyoD.Group + datReg$Myf5.Group)
Residuals:

Min	1Q	Median	3Q	Max
-16.694	-5.659	-2.415	4.504	28.098

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	19.649	7.186	2.734	0.0121
datReg$Fib..Type.Ratio	6.612	6.885	0.96	0.3473
datReg$MyoD.Group	-2.059	3.059	-0.673	0.5078
datReg$Myf5.Group	1.847	2.424	0.762	0.4541

Residual standard error: 10.14 on 22 degrees of freedom
(37 observations deleted due to missingness)
Multiple R-squared: 0.05833, Adjusted R-squared: **-0.07008**
F-statistic: 0.4542 on 3 and 22 DF, p-value: 0.7169

12-week Daily Step Count

Call:
lm(formula = datReg$Daily.Step.Count..12W. ~ datReg$CDK2NA.Group)
Residuals:

Min	1Q	Median	3Q	Max
-2647.9	-1061.6	-272.1	367.6	7909.4

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	5065	1352	3.747	0.00137
datReg$CDK2NA.Group	-1023	1095	-0.935	0.36176

Residual standard error: 2341 on 19 degrees of freedom
(42 observations deleted due to missingness)
Multiple R-squared: 0.04394, Adjusted R-squared: **-0.006375**
F-statistic: 0.8733 on 1 and 19 DF, p-value: 0.3618

12-month Daily Step Count

Call:
lm(formula = datReg$Daily.Step.Count..12M. ~ datReg$Myog.Group)
Residuals:

Min	1Q	Median	3Q	Max
-4362.4	-1112.2	-215.4	1231	7140.6

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	6901.2	1185	5.824	8.83E-06
datReg$Myog.Group	-644.5	330.9	-1.948	0.0649

Residual standard error: 2633 on 21 degrees of freedom
(40 observations deleted due to missingness)
Multiple R-squared: 0.153, Adjusted R-squared: **0.1127**
F-statistic: 3.794 on 1 and 21 DF, p-value: 0.06493

12-week KOOS5 PROM Score

Call:
lm(formula = datReg$KOOS5.Index..12W. ~ datReg$CDK2NA.Group +
datReg$IL6.Group)
Residuals:

Min	1Q	Median	3Q	Max
-37.86	-10.72	2.34	13.64	25.09

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	63.971	9.591	6.67	6.90E-08
datReg$CDK2NA.Group	6.972	4.385	1.59	0.12
datReg$IL6.Group	-7.333	5.617	-1.306	0.2

Residual standard error: 17.8 on 38 degrees of freedom
(22 observations deleted due to missingness)
Multiple R-squared: 0.1018, Adjusted R-squared: **0.05448**
F-statistic: 2.152 on 2 and 38 DF, p-value: 0.1302

12-month KOOS5 PROM Score

Call:
lm(formula = datReg$KOOS5.Index..12M. ~ datReg$Myog.Group + datReg$TNF.Group)
Residuals:

Min	1Q	Median	3Q	Max
-48.155	-8.287	-0.817	10.725	33.073

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	65.774	7.217	9.114	1.66E-11
datReg$Myog.Group	4.299	1.612	2.667	0.0108
datReg$TNF.Group	-7.106	3.577	-1.986	0.0535

Residual standard error: 17.65 on 42 degrees of freedom
(18 observations deleted due to missingness)
Multiple R-squared: 0.1827, Adjusted R-squared: **0.1437**
F-statistic: 4.693 on 2 and 42 DF, p-value: 0.01447

Baseline Demographic, Lifestyle, and Comorbidity Contributing Factors to Patient Physiology

Pax7

Call:
lm(formula = datReg$Pax7.Group ~ datReg$Age.Groups + datReg$Dominant.Leg.Operated +
datReg$Co.morb..Diab. + datReg$DHx.NSAIDs)

Residuals:

Min	1Q	Median	3Q	Max
-2.6765	-0.7889	0.1727	0.9077	1.8919

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.78776	0.48709	5.723	6.62E-07
datReg$Age.Groups	0.20791	0.08201	2.535	0.0146
datReg$Dominant.Leg.Operated	-0.71921	0.33893	-2.122	0.039
datReg$Co.morb..Diab.	-1.00705	0.51606	-1.951	0.0569
datReg$DHx.NSAIDs	0.6808	0.39682	1.716	0.0927

Residual standard error: 1.231 on 48 degrees of freedom
(10 observations deleted due to missingness)
Multiple R-squared: 0.2668, Adjusted R-squared: **0.2057**
F-statistic: 4.367 on 4 and 48 DF, p-value: 0.004309

MyoD

Call:
lm(formula = datReg$MyoD.Group ~ datReg$Age.Groups + datReg$Arth..Hx.Contra..Knee +
datReg$DHx.NSAIDs + datReg$Alcohol.Hx)

Residuals:

Min	1Q	Median	3Q	Max
-1.41793	-0.54738	-0.03453	0.45262	1.58835

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.01372	0.37948	2.671	0.0102
datReg$Age.Groups	0.08766	0.04738	1.85	0.0702
datReg$Arth..Hx.Contra..Knee	-0.35141	0.2079	-1.69	0.0972
datReg$DHx.NSAIDs	0.47184	0.23157	2.038	0.0469
datReg$Alcohol.Hx	0.22339	0.11153	2.003	0.0506

Residual standard error: 0.7262 on 50 degrees of freedom
(8 observations deleted due to missingness)
Multiple R-squared: 0.2102, Adjusted R-squared: **0.147**
F-statistic: 3.326 on 4 and 50 DF, p-value: 0.01717

Myf5

Call:
lm(formula = datReg$Myf5.Group ~ datReg$Co.morb..Hyp..Ten. +
datReg$Arth..Hx.Contra..Knee + datReg$DHx.Paracetamol + datReg$DHx.NSAIDs +
datReg$Man..Lab..Job.Hist.)

Residuals:

Min	1Q	Median	3Q	Max
-0.70502	-0.50294	-0.2987	0.06062	2.31177

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.26433	0.28165	4.489	4.22e-05 ***
datReg$Co.morb..Hyp..Ten.	0.25541	0.23859	1.07	0.29
datReg$Arth..Hx.Contra..Knee	-0.2692	0.22739	-1.184	0.242
datReg$DHx.Paracetamol	0.18365	0.24567	0.748	0.458
datReg$DHx.NSAIDs	0.18529	0.26331	0.704	0.485
datReg$Man..Lab..Job.Hist.	0.06875	0.23423	0.294	0.77

Residual standard error: 0.7906 on 50 degrees of freedom
(7 observations deleted due to missingness)
Multiple R-squared: 0.06852, Adjusted R-squared: **-0.02463**
F-statistic: 0.7356 on 5 and 50 DF, p-value: 0.6003

Myog

Call:
lm(formula = datReg$Myog.Group ~ datReg$Age.Groups + datReg$Alcohol.Hx)
Residuals:

Min	1Q	Median	3Q	Max
-3.3506	-1.3336	0.2453	1.2104	2.5169

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.71148	0.68912	1.032	0.30655
datReg$Age.Groups	0.24544	0.09838	2.495	0.01575
datReg$Alcohol.Hx	0.64035	0.23413	2.735	0.00847

Residual standard error: 1.535 on 53 degrees of freedom
(7 observations deleted due to missingness)
Multiple R-squared: 0.2075, Adjusted R-squared: **0.1776**
F-statistic: 6.939 on 2 and 53 DF, p-value: 0.002105

CDK2NA

Call:
lm(formula = datReg$CDK2NA.Group ~ datReg$Age.Groups + datReg$Arth..Hx.Contra..Knee)
Residuals:

Min	1Q	Median	3Q	Max
-0.86213	-0.29944	-0.13563	0.08945	1.47548

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.84929	0.18981	4.474	3.99E-05
datReg$Age.Groups	0.11254	0.03208	3.508	0.000917
datReg$Arth..Hx.Contra..Knee	-0.27635	0.13255	-2.085	0.041828

Residual standard error: 0.4868 on 54 degrees of freedom
(6 observations deleted due to missingness)
Multiple R-squared: 0.2677, Adjusted R-squared: **0.2406**
F-statistic: 9.872 on 2 and 54 DF, p-value: 0.0002219

IL6

Call:
lm(formula = datReg$IL6.Group ~ datReg$Age.Groups + datReg$BMI.Groups +
datReg$Co.morb..Diab. + datReg$Peak.Phys..Act..Tegner + datReg$SIMD.Quint)
Residuals:

Min	1Q	Median	3Q	Max
-0.6876	-0.3769	-0.1353	0.1461	1.2673

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.38064	0.42082	3.281	0.00195
datReg$Age.Groups	0.07632	0.03809	2.004	0.05088
datReg$BMI.Groups	-0.13185	0.06425	-2.052	0.04574
datReg$Co.morb..Diab.	0.32226	0.24406	1.32	0.19309
datReg$Peak.Phys..Act..Tegner	0.03942	0.02919	1.351	0.18322
datReg$SIMD.Quint	-0.10257	0.06207	-1.653	0.1051

Residual standard error: 0.5341 on 47 degrees of freedom
(10 observations deleted due to missingness)
Multiple R-squared: 0.236, Adjusted R-squared: **0.1547**
F-statistic: 2.903 on 5 and 47 DF, p-value: 0.02302

TNF

Call:
lm(formula = datReg$TNF.Group ~ datReg$Age.Groups + datReg$Arth..Hx.Contra..Knee +
datReg$DHx.NSAIDs)
Residuals: s:

Min	1Q	Median	3Q	Max
-0.9965	-0.5121	-0.2324	0.4389	1.8395

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.23738	0.27153	4.557	3.01E-05
datReg$Age.Groups	0.07195	0.04435	1.622	0.1106
datReg$Arth..Hx.Contra..Knee	-0.36469	0.1897	-1.922	0.0598
datReg$DHx.NSAIDs	0.39935	0.21425	1.864	0.0678

Residual standard error: 0.6962 on 54 degrees of freedom
(5 observations deleted due to missingness)
Multiple R-squared: 0.1398, Adjusted R-squared: **0.09201**
F-statistic: 2.925 on 3 and 54 DF, p-value: 0.04194

Fibre Type Ratio

Call:
lm(formula = datReg$Fib..Type.Ratio ~ datReg$Sex + datReg$Dominant.Leg.Operated +
datReg$DHx.Paracetamol + datReg$SIMD.Quint)

Residuals:

Min	1Q	Median	3Q	Max
-0.67821	-0.15617	-0.00434	0.11051	0.73845

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.81524	0.1199	6.8	2.25E-08
datReg$Sex	-0.16818	0.07311	-2.3	0.026235
datReg$Dominant.Leg.Operated	0.14298	0.07275	1.965	0.055691
datReg$DHx.Paracetamol	-0.31422	0.0877	-3.583	0.000845
datReg$SIMD.Quint	0.04666	0.02824	1.652	0.105577

Residual standard error: 0.2529 on 44 degrees of freedom
(14 observations deleted due to missingness)
Multiple R-squared: 0.3247, Adjusted R-squared: **0.2633**
F-statistic: 5.29 on 4 and 44 DF, p-value: 0.001443

Type 1 Fibre Diameter

Call:
lm(formula = datReg$Type.1.Fib..Diam. ~ datReg$Age.Groups + datReg$Arth..Hx.Contra..Knee +
datReg$DHx.NSAIDs + datReg$Peak.Phys..Act..Tegner)

Residuals:

Min	1Q	Median	3Q	Max
-21.2617	-5.8431	-0.6311	6.2054	24.5262

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	80.2624	7.3178	10.968	2.66E-14
datReg$Age.Groups	-2.0478	0.7363	-2.781	0.00788
datReg$Arth..Hx.Contra..Knee	-7.1156	3.3728	-2.11	0.04047
datReg$DHx.NSAIDs	6.237	3.4098	1.829	0.07401
datReg$Peak.Phys..Act..Tegner	-1.3479	0.6209	-2.171	0.03523

Residual standard error: 10.48 on 45 degrees of freedom
(13 observations deleted due to missingness)
Multiple R-squared: 0.2697, Adjusted R-squared: **0.2048**
F-statistic: 4.155 on 4 and 45 DF, p-value: 0.005996

Type 2 Fibre Diameter

Call:
lm(formula = datReg$Type.2.Fib..Diam. ~ datReg$BMI.Groups + datReg$BEI.Groups +
datReg$SIMD.Quint)

Residuals:

Min	1Q	Median	3Q	Max
-23.717	-6.753	-1.978	6.043	27.413

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	74.725	8.209	9.103	2.15E-11
datReg$BMI.Groups	3.581	2.274	1.575	0.123
datReg$BEI.Groups	-2.822	1.324	-2.131	0.0391
datReg$SIMD.Quint	-2.471	1.456	-1.697	0.0973

Residual standard error: 12.32 on 41 degrees of freedom
(18 observations deleted due to missingness)
Multiple R-squared: 0.1751, Adjusted R-squared: **0.1148**
F-statistic: 2.902 on 3 and 41 DF, p-value: 0.04627

Overarching Combined Multivariate Model Contributing Factors to Functional Surgical Outcomes

12-month ALF Timed Functional Score

Call:
lm(formula = datTert$ALF.Score..12M. ~ datTert$Alcohol.Hx + datTert$ALF_PAC)
Residuals:

Min	1Q	Median	3Q	Max
-8.8339	-2.9548	-0.9338	2.1901	24.2291

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-8.1582	4.7311	-1.724	0.09493
datTert$Alcohol.Hx	4.3411	1.3649	3.181	0.00341
datTert$ALF_PAC	0.7982	0.1259	6.338	5.45E-07

Residual standard error: 6.41 on 30 degrees of freedom
(30 observations deleted due to missingness)
Multiple R-squared: 0.6288, Adjusted R-squared: **0.6041**
F-statistic: 25.41 on 2 and 30 DF, p-value: 3.497e-07

12-week Daily Step Count

Call:
lm(formula = datTert$Daily.Step.Count..12W. ~ datTert$Age.Groups +
datTert$FJS_PAC)
Residuals:

Min	1Q	Median	3Q	Max
-3824.2	-859.4	-7.8	937.2	3594.3

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	4945.29	978.6	5.053	5.28E-05
datTert$Age.Groups	-532.82	204.63	-2.604	0.016577
datTert$FJS_PAC	99.79	23.55	4.237	0.000369

Residual standard error: 1628 on 21 degrees of freedom
(39 observations deleted due to missingness)
Multiple R-squared: 0.5373, Adjusted R-squared: 0.4932
F-statistic: 12.19 on 2 and 21 DF, p-value: 0.0003061

12-month Daily Step Count

Call:
lm(formula = datTert$Daily.Step.Count..12M. ~ datTert$DHx.Paracetamol +
datTert$Co.morb..Hyp..Ten. + datTert$Smoking.Hx + datTert$ALF_PAC)
Residuals:

Min	1Q	Median	3Q	Max
-3414.7	-1474.3	-223.1	1284	5724

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	7690.96	1864.49	4.125	0.000576
datTert$DHx.Paracetamol	2199.14	1183.17	1.859	0.078634
datTert$Co.morb..Hyp..Ten.	-2047.83	1005.21	-2.037	0.055803
datTert$Smoking.Hx	-2045.83	1068.52	-1.915	0.07072
datTert$ALF_PAC	-110.12	56.03	-1.965	0.064157

Residual standard error: 2216 on 19 degrees of freedom
(39 observations deleted due to missingness)
Multiple R-squared: 0.4991, Adjusted R-squared: 0.3937
F-statistic: 4.734 on 4 and 19 DF, p-value: 0.00806

12-month KOOS5 PROM Score

Call:
lm(formula = datTert$KOOS5.Index..12M. ~ datTert$K.L.OA.Score +
datTert$Myog.Group)
Residuals:

Min	1Q	Median	3Q	Max
-41.347	-10.914	1.665	11.213	31.837

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	41.251	7.117	5.796	7.79E-07
datTert$K.L.OA.Score	9.887	2.917	3.389	0.00153
datTert$Myog.Group	3.179	1.475	2.156	0.03688

Residual standard error: 16.36 on 42 degrees of freedom
(18 observations deleted due to missingness)
Multiple R-squared: 0.2979, Adjusted R-squared: 0.2645
F-statistic: 8.91 on 2 and 42 DF, p-value: 0.0005949

Indicative Directional Model Factor Contributions Charts

Response		Contributing Factor																																		
		Sex	Patient age at time of surgery	Body Mass Index at time of surgery	Bioelectrical impedence at time of surgery	Kellgren and Lawrence Radiographic Osteoarthritis Score	If the patient's operated knee was their dominant leg	Comorbidity - diabetes	Comorbidity - hypertension	History of previous arthroplasty in contralateral knee	Long term pharamaceutical use - paracetamol	Long term pharamaceutical use - NSAIDs	Long term pharamaceutical use - opiates	Occupational history	Patient alchohol group	Patient smoking history	Patient peak regular activity level in life	Scottish Index of Multiple Deprivation Quintile	Fibre Type Ratio	Type 1 Fibre Diameter	Type 2 Fibre Diameter	Pax7	MyoD	Myf5	Myog	CDK2NA	IL6	TNF
Nottingham Leg Rig Power Ratio (W/kg)	12W													-			+																		
	12M											+				-							+			-									
Aggregated Locomotor Function Score (seconds)	12W																																			
	12M												+	+	+			-																		
Average daily step count (steps)	12W		-																+																	
	12M								-		+					-										-									
Knee injury and Osteoarthritis Outcome Score Index (KOOS5)	12W					+		-																												
	12M	-		-		+							-					-									+		-						

Response		Contributing Factor																																		
		Sex	Patient age at time of surgery	Body Mass Index at time of surgery	Bioelectrical impedence at time of surgery	Kellgren and Lawrence Radiographic Osteoarthritis Score	If the patient's operated knee was their dominant leg	Comorbidity - diabetes	Comorbidity - hypertension	History of previous arthroplasty in contralateral knee	Long term pharamaceutical use - paracetamol	Long term pharamaceutical use - NSAIDs	Long term pharamaceutical use - opiates	Occupational history	Patient alchohol group	Patient smoking history	Patient peak regular activity level in life	Scottish Index of Multiple Deprivation Quintile																
Myogenesis: Pax7			+				-	-				+																								
Myogenesis: MyoD			+							-		+			+																					
Myogenesis: Myf5																																				
Myogenesis: Myog			+												+																					
Senescence: CDK2NA			+							-																										
Inflammation: IL6			+	-				+									+	-																		
Inflammation: TNF			+							-		+																								
Histology: Fibre Type Ratio		-					+				-							+																		
Histology: Type 1 Fibre Diameter			-							-		+					-																			
Histology: Type 2 Fibre Diameter				+	-													-																		

Appendix D: Conference Paper Abstracts

British Orthopaedic Research Society (BORS) 2018

University of Leeds, United Kingdom
10-11th September 2018

Relationship Between Differing Measures of Function Following Total Knee Arthroplasty

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Background

The first three months following Total Knee Arthroplasty (TKA) provide an early window into a patient's functional outcomes. The aim of this study was to explore how different measures change in the early post-op window and relate to each other.

Methods

20 patients due to undergo primary TKA were recruited prospectively. Data were recorded at three time points; pre-operation, 6 and 12-weeks post-operation. Patient function was monitored using a variety of metrics at each time point; direct functional testing of lower limb power (Nottingham Rig), timed functional performance of daily tasks (Aggregated locomotor function score), activity monitoring (step count), and patient reported function (KOOS ADL sub-score). Data analysis: Two-way ANOVAs, Correlation Coefficients.

Results

Compared to pre-op data; power output was similar at 6Wks ($p = 0.37$) but increased by 12Wks ($p < 0.05$). Daily step count significantly decreased at 6Wks ($p < 0.05$), then recovered to baseline levels by 12 weeks ($p = 0.30$). Timed performance remained similar across all three time points ($p = 0.27$). Patient reported activities of daily living significantly increased at 6Wks ($p < 0.05$) with no further change at 12Wks ($p = 0.10$).

Significant changes in metric since last assessment:

	Pre-op to 6 weeks	6 weeks to 12 weeks
Power output	-	↑
Step count	↓	↑
Timed performance	-	-
Patient reported function	↑	-

Moderate to strong correlations were seen across all measures post-operatively; power output and timed performance ($r = 0.62$), timed performance and patient reported function ($r = 0.61$), power output and patient reported function ($r = 0.49$), timed performance and activity monitoring ($r = 0.37$).

Conclusions

We demonstrate substantial variation in the course of improvement of different tools. As such, the use of a single functional measure limits comparative evaluation against alternative measures across studies. This has major implications for the design of clinical studies and the interpretation of data obtained from different trials at varying timepoints post-surgery.

European Orthopaedic Research Society (EORS) 2018
Galway, Ireland
25-28th September 2018

VARIATION IN EARLY FUNCTIONAL OUTCOME MEASUREMENTS FOLLOWING TOTAL KNEE ARTHROPLASTY

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The first three months following Total Knee Arthroplasty (TKA) provide an early window into a patient's functional outcomes, with the change of function in this time yielding valuable insight.
20 patients due to undergo primary TKA were recruited to the study. Data were recorded at three time points; pre-assessment clinic (PAC) before the operation, 6-weeks-post-operation (6WKs), at 12-weeks-post-operation (12WKs).

Functional activity levels were monitored during early post-operative recovery for changes in early functional outcome, and allowed a comparison of metrics at each time point. This included direct functional testing of power output, timed functional performance in clinic, patient reported outcome measures, and multiday activity monitoring devices.

Maximal power output symmetry (Power) was similar at 6WKs vs PAC ($p = 0.37$). At 12WKs, it had increased ($p < 0.05$). Timed functional performance (Performance) remained similar across all three time points ($p = 0.27$). Patient reported activities of daily living (ADL) performance significantly increased at 6WKs vs PAC ($p < 0.05$). At 12WKs, it remained similar ($p = 0.10$). Patient daily step count significantly decreased at 6WKs vs PAC ($p < 0.05$). By 12WKs, this had increased to similar levels to PAC ($p = 0.30$).

Within the functional outcome measures, strong post-operative correlations were observed between Power and Performance ($r = 0.62$), Power and ADL ($r = 0.49$), and Performance and ADL ($r = 0.61$).

Despite reduced measured step count and similar functional performance, patients report improved ADL at 6WKs. When symmetrical power output and measured step count have improved at 12WKs, patients report similar ADL to that at 6WKs. Multiple measures are required to get a full picture, however this highlights the different aspects measured by different tools.

Orthopaedic Research Society (ORS) 2019
Austin, TX, United States of America
2-5th February 2019

Patient Functional Ability Improves Following Total Knee Arthroplasty but Pre-Operative Activity Pattern Remains

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Disclosures: MTA Griffin (None), AHRW Simpson (5-Stryker, Corin, Pfizer, 8-Bone and Joint Research, 9-BOA, BORS), DF. Hamilton (2-Stryker, 5-Stryker, 8-BMC Musculoskeletal Disorders)

INTRODUCTION: A key facet of patient outcome following total knee arthroplasty is the restoration of physical function. Various methods can be used to measure this outcome; Patient reported outcome measures (PROMs), or more direct evaluations of strength assessments, timed activities, or biometric measurements. More recently activity monitors have been employed as an effective way to capture patients function without the reliance of clinic or laboratory based assessments. There is however little understanding of the interrelationship between these various ways of measuring the patient's ability to perform physical activity. Our aim was to evaluate the effectiveness of take-home activity monitoring devices and how the functional metric of step-count correlated with established clinic-based functional assessments of outcome.

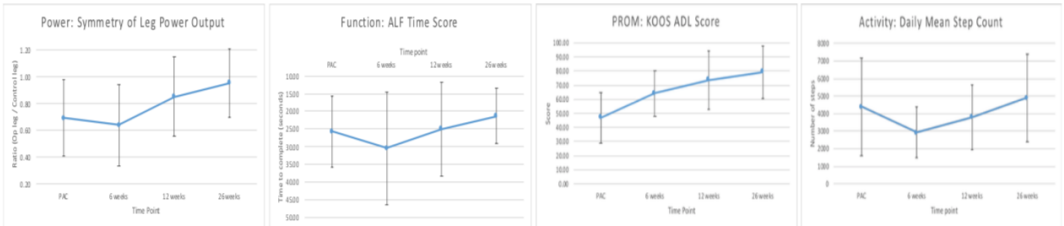
METHODS: Following local approvals, 20 patients due to undergo primary TKA were prospectively recruited and consented to attend pre- and post-op research clinics. Data were recorded at four time points; pre-operation, 6-, 12-, and 26-weeks post-operation. Patient functional activity levels were monitored with a battery of functional metrics. Lower limb power output was assessed with the Leg Extensor Power Rig (Nottingham, UK), reported as a ratio of control limb acting as an internal control. Timed functional performance was assessed with the Aggregated Locomotor Function (ALF) score, a composite of walking, chair transfer and stair climb (lower scores highlight superior function). Patient reported function was assessed with the Knee injury and Osteoarthritis Outcome Score Activities of Daily Living sub-score (KOOS ADL). Multiday activity monitoring devices (Xiaomi MiBand 2) counted steps over 3 consecutive days and were reported as a daily average value. Analysis was by Two-way ANOVA and Correlation Coefficients, with statistical significance accepted at 0.05.

RESULTS: Compared to pre-op, by 26 weeks patients had made significant improvements in proportional lower limb power (mean change 69% to 96%; $p<0.05$), ALF score (mean change: 25.7 to 21.2 secs; $p<0.05$); KOOS ADL (mean change: 46.99 to 79.41; $p<0.05$), however and step count stayed similar to pre-operative levels (mean change: 4403 to 4898 steps, $p=0.29$). Figure 1. The functional metrics demonstrate different trajectories of change across the assessment time points. Proportional power output was similar to pre-op levels at 6 weeks ($p=0.37$) then improved by 12 week ($p<0.05$) and 26 weeks ($p<0.05$). ALF score remained similar to pre-op values 6 and 12 weeks ($p = 0.27$) then improved by 26 weeks ($p<0.05$). Patient reported function increased by 6 weeks ($p<0.05$), followed by a non-significant increase at 12 weeks ($p= 0.10$), and further increase at 26 weeks ($p<0.05$). Step count decreased from pre-op to 6 weeks ($p<0.05$), with no further change by 12 weeks ($p=0.20$), then increased back to baseline levels at 26 weeks ($p<0.05$). Figure 1. Modest to strong correlations were observed across all measurements highlighting the interconnected nature of these varying functional assessments; lower limb power and ALF ($r=0.52$), lower limb power and KOOS ADL ($r=0.52$), ALF and KOOS ADL ($r=0.50$), and ALF and step count ($r=0.38$). A notable weak correlation was observed at the 12 weeks post-op time point between ALF score and average daily step count ($r=0.14$), compared to the strong correlations at 6 weeks ($r=0.52$) and 26 weeks ($r=0.54$).

DISCUSSION: The outcome measurements showed different trends across the four time points. Lower limb power, ALF score, and step count initially deteriorated before improving, whereas patient reported function increased by the first post-op time point and continued to do so by 26 weeks. This difference in patient perceived performance and directly measured performance highlights the different nature of these assessments. While lower limb power, ALF score, and patient reported function were all improved at 26 weeks compared to pre-op values, step count had only returned to a similar value. Interestingly, while patients may have the physiological ability to extend their range and increase their step count based on improved strength and perceived ability, it seems that they choose not to do so based on established lifestyle and activity habits. The weaker correlation at 12 weeks between ALF and step count was notable as it may reflect the improvement in strength and ability to perform representative ADLs quickly, but that the endurance is still lacking for activities such as walking distance. This endurance may have recovered by the 26 week time point resulting in the improved correlation with the other metrics. Further data collection at subsequent time points may provide greater insight as to this. While not directly measured in this study, complimentary step count data such as step cadence or speed may further elucidate the picture in comparing walking ability and endurance between pre- and post-op function. Movement quality may have improved substantially, though it seems distance has not. Different sensitivity to recovery was observed across the range of functional outcome measurements. This reinforces the importance of multiple tool use in the design of clinical studies, to reflect the differing aspects of function, and that careful analysis is required due to the variation in metrics at different time-points.

SIGNIFICANCE: Patients are able to demonstrate greater functional ability in tests of maximal capacity following total knee arthroplasty, but they demonstrate habitual levels of activity, consistent with pre-operative values suggestion that activity behaviour modification may be required to utilise the physical benefit of knee arthroplasty.

FIGURE 1 – Functional metrics across time points. Error bars indicate standard deviations.



International Combined Meeting of Orthopaedic Research Societies (ICORS) 2019
Montréal, Canada
19-22nd June 2019

Patient Function Improves Following TKA but Activity Levels Remain at Pre-op Values

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PURPOSE: Various methods can be used to measure functional ability following Total Knee Arthroplasty (TKA). Patient reported outcome measures (PROMs), or more direct assessments can be used. There is little understanding of the interrelationship between these various ways of measuring the patient's physical activity. Our aim was to evaluate the effectiveness of take-home activity monitoring devices and how the functional metric of step-count correlated with established clinic-based functional assessments of outcome.

METHODS: 20 patients due to undergo primary TKA were prospectively recruited. Data were recorded at four time points; pre-operation, 6-, 12-, and 26-weeks post-operation. Patient functional activity levels were monitored with four metrics. Lower limb power output was assessed with the Leg Extensor Power Rig. Timed functional performance was assessed with the Aggregated Locomotor Function (ALF) score. Patient reported function was assessed with the Knee injury and Osteoarthritis Outcome Score Activities of Daily Living sub-score (KOOS-ADL). Multiday activity monitoring devices (Xiaomi MiBand 2) counted steps over 3 consecutive days and were reported as a daily average value. Analysis was by Two-way ANOVA and Correlation Coefficients, with statistical significance accepted at 0.05.

RESULTS: Compared to pre-op, by 26-weeks patients had significantly improved lower limb power ($p<0.05$), ALF score ($p<0.05$); KOOS ADL ($p<0.05$), however step count stayed similar to pre-operative levels ($p=0.29$). Proportional power output was similar to pre-op levels at 6-weeks ($p=0.37$) then improved by 12-weeks ($p<0.05$) and 26-weeks ($p<0.05$). ALF score remained similar to pre-op values 6- and 12-weeks ($p = 0.27$) then improved by 26-weeks ($p<0.05$). Patient reported function increased by 6-weeks ($p<0.05$), followed by a non-significant increase at 12-weeks ($p= 0.10$), and further increase at 26-weeks ($p<0.05$). Step count decreased from pre-op to 6-weeks ($p<0.05$), with no further change by 12-weeks ($p=0.20$), then increased back to baseline levels at 26-weeks ($p<0.05$). Modest to strong correlations were observed across all measurements; lower limb power and ALF ($r=0.52$), lower limb power and KOOS ADL ($r=0.52$), ALF and KOOS ADL ($r=0.50$), and ALF and step count ($r=0.38$).

CONCLUSION: The results highlight the variation in functional measures over time in TKA. Additionally, while lower limb power, ALF score, and patient reported function were all improved at 26-weeks compared to pre-op values, step count had only returned to a similar value. While patients may have the physiological ability to increase their step count based on improved strength and perceived ability, they seem to choose not to do so based on established lifestyle and activity habits. Patients are able to demonstrate greater functional ability in tests of maximal capacity following total knee arthroplasty, but they demonstrate habitual levels of activity, consistent with pre-operative values suggestion that activity behaviour modification may be required to utilise the physical benefit of knee arthroplasty.

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